

SHORT THESIS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY
(PHD)

The role of UV-induced DNA damage response and zinc homeostasis in
dermatological disorders

by

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The examination takes place at the Conference room of Faculty of Public Health, University of Debrecen, at 11 am, November 23, 2015

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INTRODUCTION

The structure and function of human skin

The skin is the most external part of our body, it forms 15-20% of the total body weight and its surface is 1.6-2.3 m². It includes three main structures: the epidermis, the dermis and subcutis. The epidermis is the external layer of skin and has ectodermal origin. It is mostly composed of keratinocytes going through a vertical differentiation process, forming a multi-layered stratified squamous epithelium. The final steps of keratinocyte differentiation lead to the formation of the stratum corneum, the most external barrier against environmental aggressions. The epidermis can be divided into five layers: stratum basale, stratum spinosum, stratum granulosum, stratum lucidum, stratum corneum. The epidermis also includes melanocytes, Langerhans cells and Merkel cells. Melanocytes are dispersed located between the cells of stratum basale, show dendritic cell morphology, have neuroectoderm origin and responsible for the production of melanin pigments. Langerhans cells, characterized by Birbeck-granules, are located in stratum basale and spinosum. These cells have mesenchymal origin and are members of the monocyte-macrophage system as antigen presenting cells. Merkel cells are located in the stratum basale and interacting with nerve endings. The dermis is the area of supportive connective tissue between the epidermis and the underlying subcutis with a thickness of 3 mm. It is made up of two layers: stratum papillare, stratum reticulare. The subcutis is the layer of loose connective tissue and fat beneath the dermis. It also includes blood vessels and nerves. The skin performs multiple functions, such as protection, homeostasis, sensory organ, secretion, excretion and thermal insulation, nutrient storage and immunological regulation.

Solar UV light irradiation, photodamage and its biological consequences, clinical aspects of UV-induced skin damage, short term effects, long term effects of skin photo-damage, non-melanoma skin cancers and melanoma

Solar UV exposure is one of the most important environmental factors affecting skin physiology. UV radiation, expanding the 200 to 400 nm wavelengths of the electromagnetic spectrum, is a high-energy component of solar radiation. UVR is divided into three ranges based on wavelength. 1-10 % of the UVB (320–290 nm) radiation reaches the earth's surface. Wavelength in the UVB range is mainly absorbed by epidermal cells. Its toxic effects are mainly mediated by the direct absorption of photons by DNA. It causes DNA damage by the formation of dimeric photoproducts between adjacent pyrimidine bases on the same strand.

Two forms of pyrimidine dimers are cyclobutane pyrimidine dimer (CPD) and pyrimidine (6-4) pyrimidone photoproducts (PP). In addition UVB can damage proteins or cell membranes by generating oxidative stress. In response to internal and external stress such as genomic DNA damage upon UV irradiation and suboptimal growth conditions, proliferating cells trigger mechanisms called cell cycle checkpoints that assess and respond to the stress. To combat the diversity of DNA lesions, cells have evolved a complex DNA damage response (DDR) that can engage many different DNA repair pathways, including nucleotide excision repair (NER) by which CPDs and (6-4) PPs are repaired. Checkpoint-arrested cells resume cell-cycle progression once damage has been repaired, whereas cells with irreparable DNA lesions undergo permanent cell-cycle arrest or cell death.

1. Apoptosis: there is intrinsic pathway of apoptosis, in which reactive oxygen species (ROS) generated upon UV irradiation have been recognized to serve as initiators, which causes mitochondrial damage. UV trigger cell death receptors (CD95/Fas, TRAIL, and TNFR1) by autocrine release of death ligands or in a ligand-independent manner. Therefore, UV additionally induces the extrinsic apoptotic pathway. Early apoptotic cells are characterized by the retained membrane integrity, in contrast late apoptotic cells are characterized by compromised membranes and act like necrotic cells, which also lack intact membranes, possibly because of the leakage of pro-inflammatory intracellular contents.
2. Necrosis: it is a non-apoptotic cell death. Apoptosis is a silent form of cell death, the intact cell disappears through phagocytic uptake, it can be considered to be immunosuppressive. In contrast necrosis is usually related to an earlier breakdown of plasma-membrane integrity, which may permit the release of pro-inflammatory cellular components, it can be considered to be immunostimulatory. Therefore balance between the two processes plays a substantial role in the decision of whether the uptake of dead cells causes a productive immune response or not. Other processes which are involved in cell death pathways are autophagy and senescence.

Sunburn (erythema), immunosuppression and suntan (pigmentation) are the immediate responses of normal human skin exposed to UVR; these are the short term effects of skin damage. UVB is the most effective waveband for inducing erythema. UVB can induce immunosuppression and melanogenesis called tanning response. Regarding the long-term effects, UVB portion of the sunlight has strong effectiveness on photo-carcinogenesis. There are epidemiologic and molecular evidences connected all forms of skin cancer to UV, and UV is causative factor for approximately 65% of melanoma and 90% of non-melanoma skin

cancers. UVB represent carcinogens for non-melanoma skin cancer, including squamous cell carcinoma (SCC) and basal cell carcinoma (BCC) and are emerging as causative factors in malignant melanoma (MM) formation. Cutaneous Malignant Melanoma (CMM) is the most dangerous form of skin cancer, with high risk of metastasis. Malignant melanomas are developed with high risk from dysplastic nevi, which are characterized with appearance at a young age. CMMs are described with four types: Superficial spreading melanoma (SSM), Nodular melanoma (NM), Acral lentiginous melanoma and Lentigo maligna melanoma. To characterize the widespread of the tumor and to find out the risk for metastasis an evidence-based staging system is used. It is determined by the tumor-lymph node-metastasis (TNM) classification for tumor staging according to 2009 American Joint Committee on Cancer (AJCC) guidelines. It includes primary tumor thickness, primary tumor ulceration and primary tumor mitotic rate and previously Clark level was also included to the staging criterions. The AJCC clinicopathological staging system for the prognosis of CMM assigns patients to risk categories, but it does not predict individual patient outcomes because of heterogeneity of the tumor.

Promising prognostic biomarkers for the prognosis of CMM, CMM in the aspect of tumor immunology

Immunohistochemically detectable tissue-based prognostic protein biomarkers: Several potential protein biomarkers have been identified, which show statistically significant association with melanoma-specific mortality and disease-free patient survival. Promising protein biomarkers with independent prognostic significance from clinicopathological parameters are AP-2, ATF-2, NCOA3, PRKCA, Bcl-2, Survivin, CEACAM-1, CXCR4, CD44, MCAM, L1-CAM, MMP2, OPN, Tenascin-C, tPA, HMB45, iNOS, p16/INK4A, p27, CyclinA, MAP-2, Metallothionein (MT), p53, Ki67, bFGF, β -Catenin, Bcl-6, Dysadherin, HNK-1, HIF2 α , GADD153, Melastatin, MITF, p-Akt, RGS1, RUNX3, RBM3, PUMA and PHH3. However none of these is introduced in routine clinical practice, because these studies need validation.

Besides heterogeneity, CMM can also be characterized by its immunogenicity. There are number of clinical studies related to the activity of the immune system in melanoma, and these observations also provide strong evidences that the immune system can naturally react to and destroy or control melanoma. During progression, the tumor remodels the microenvironment. The crosstalk between the melanoma cells and the surrounding cells promote the metastatic process by inducing angiogenesis, invasion, migration, and

colonization in a secondary organ. There is a need for a more accurate characterization of CMMs including the tumor microenvironment for a better understanding of the individual behaviour of the tumor and to perform a patient-targeted therapy.

Role of zinc homeostasis in skin

Zinc is an essential microelement, it has critical role in normal health and development. Zinc content of the body is associated with skin by approximately 9%, primarily with the epidermis (50–70 $\mu\text{g g}^{-1}$ dry weight). Its importance in skin is shown by the severe skin symptoms of hereditary or acquired zinc deficiency, including erythematous rashes, scaly plaques, ulcers, and the genetic inability to absorb zinc from the intestine results in acrodermatitis enteropathica, which manifests itself as severe dermatitis at orifices and acra. By the ability of systemic or topical zinc preparations zinc improves hair loss, acne and several inflammatory skin conditions.

The cellular homeostasis of zinc is mediated by ZIPs (Zrt-, Irt-like proteins), which family contains 14 proteins that transport zinc to the cytosol, and ZnTs, containing 10 proteins which transport zinc out of the cytosol. The prevalence of genes encoding zinc proteins is estimated to be over 3% of the 32,000 identified genes. Over 300 zinc-dependent enzymes have been defined and characterised. For example among zinc enzymes, matrix metalloproteinases participate in the hydrolyzation of structural proteins of extracellular matrix, such as collagen and elastin; zinc-finger proteins, such as DNA, RNA polymerases, regulate gene expression, by which zinc is related to the proliferation of cells, superoxide dismutase is involved in redox homeostasis with its anti-oxidant activity or alkaline phosphatase, which has role in suppressing the inflammatory process. In relation to the latter function, indeed the effect of zinc is closely related to inflammation as showed by examinations on immune cells. Zinc can also influence the cytokine release, which affects the communication among these cells. Zinc occurs in its stable ionic state of +2 in cells, it cannot participate in electron transfer chemistry, it is redox inert. Nevertheless, it has important role in reduction-oxidation processes. There is a dynamic of zinc binding to and release from proteins. In this process MT has crucial role, this characteristic of zinc is maybe connected to its role in protein functions. A close relation between redox homeostasis and the regulation of different protein functions by zinc can provide an important, fine-tuned control mechanism in the cell, but this process has been evidenced biochemically and has not been well studied in biological systems. Zinc also function as a cofactor of superoxide dismutase, and zinc deficiency increases oxidative stress.

Metallothionein, role of zinc in UV-induced DNA damage response and carcinogenesis

MTs are characterized by low molecular weight of 6-7 kDa, high cysteine content and the ability of complex of metals. One MT molecule can bind seven zinc ions and through different binding affinities to metals, it acts as a zinc muffler over several orders of magnitude. MT/thionein couple sequester or release zinc depending on the local redox state, thereby influencing the function of numerous proteins, transcription factors and enzymes. There are at least 10 isoforms of MT in human body. The major isoforms are MT I/II, which are presented in all cells. MT III and MT IV are minor isoforms and found only in specialized cells.

The action of zinc in UVB induced DNA damage response is largely unknown. It has been demonstrated that rapid intracellular elevation of zinc levels occurs in keratinocytes after UVB irradiation, and the level of MT is higher in the epidermis after acute UV exposure, while the skin of MT knockout mice is more susceptible to UVB. In addition apoptosis is observed in skin lesions of patients with acquired zinc deficiency suggesting important regulatory role of zinc in cell death pathways. An imbalance in the MT protein level can be detected in different skin cancers. Epigenetic downregulation of MT1E and MT1G has been reported recently in CMM as well. However, experimental evidence for tumor suppressor role of some MT isoforms is still lacking. Nevertheless, several types of cancer overexpress MT and the overexpression seemed to correlate with tumor progression and poor survival of patients. Increased MT I/II protein expression has been observed in keratinocyte- and melanocyte-derived tumors and it correlated with tumor invasiveness. Using whole tumor sections, MT I/II overexpression of primary CMM cells has been shown to be correlated with tumor progression, tumor thickness, invasiveness and reduced survival. Further, the overexpression of MT I/II in primary CMM cells appeared to have a higher prognostic value than that of sentinel lymph node positivity. Therefore the protein expression of MT I/II is a promising prognostic immunohistochemical factor in prognosis of CMM. However the role of MT in metastasis formation remains to be confirmed and experimental evidence for oncogenic role of certain isoforms is still lacking.

OBJECTIVES

1. Research data suggest that the up-regulation of MT expression in CMM is a significant and independent factor for reduced patient survival. However, MT has not yet become widely used as a prognostic marker because of the lack of immunohistochemical validation by different workgroups, therefore we aimed to compare the MT I/II protein expression in primary CMM samples without metastases to samples with haematogenous metastases in a retrospective manner using tissue microarray analysis (TMA) of archived paraffin tissue blocks.
2. We hypothesized that there is a relationship between the tumoral MT I/II protein expression and cells surrounding/infiltrating the tumor, thus we aimed to characterize our samples immunologically with such immunohistochemical markers that showed association with CMM prognosis previously and compare these staining patterns to alterations in tumoral MT I/II protein expression.
3. Considering the findings about the connection between MT I/II expression of tumor cells and tumor progression, we were interested in the further role of MT and zinc homeostasis in skin physiology and pathophysiology. The main environmental harmful factor to skin is UVB. We assumed that zinc homeostasis can influence the UVB-induced DNA damage response; therefore we aimed to study the effect of non-toxic Zn (II) exposure on human keratinocyte functions in conventional culture and under stressed conditions using UVB irradiation.

These findings may provide a better understanding of pathogenesis of skin diseases, and may open new therapeutical possibilities.

MATERIALS AND METHODS

Patient selection for tissue microarray

Tissue blocks were chosen from the archive of the Department of Dermatology of the University of Debrecen. Patients were selected retrospectively after considering their clinical outcome and in accordance with ethical rights. Formalin-fixed paraffin-embedded tissue blocks of primary CMM without metastases (n= 23, 1998-2003) and with hematogenous metastases (n= 23, 1998-2006) were selected for tissue microarrays (TMAs). Blocks of nevi (n= 6) were also included. Briefly, 4- μ m sections were prepared from the tissue blocks and stained with hematoxylin-eosin for validation. Thereafter, the areas of interest were selected, and multiblocks (6x10; d= 1 mm tissue cylinders/cores) were built by means of TMA MasterTM (TMA Master, USA). The serial sections that were used for immunohistochemical study were previously checked by hematoxylin-eosin staining.

Cell culture, UVB-irradiation apparatus, and ZnCl₂ treatment

HaCaT human keratinocytes were obtained from ATCC and maintained in high-glucose DMEM (PAA, Traun, Austria) supplemented with 10% fetal bovine serum ((FBS) Lonza, Verviers, Belgium), 2 mM L-glutamine (PAA, Traun, Austria), 100 U/mL penicillin G, 0.1 mg/mL streptomycin sulphate, and 0.25 μ g/mL amphotericin B (Sigma-Aldrich, St. Louis, MO, USA) in an atmosphere of 5% CO₂ at 37°C. In the UV irradiation experiments, the culture medium was replaced with sufficient phosphate-buffered saline (PBS) (DPBS, Lonza, Verviers, Belgium) to avoid dehydration. The cells were then exposed to UVB (20 mJ/cm²) using a TL20W/12 RS broadband, filtered UVB lamp (Philips, Germany), which had an emission spectrum of 280–370 nm, with a peak at 312 nm. The UV intensity was controlled with a UV light meter (UVP Inc., San Gabriel, USA). The original medium was replaced after treatment. A 0.1-M stock solution of ZnCl₂ (Sigma-Aldrich, St. Louis, MO, USA) was prepared in deionised water and sterilised with a 0.2- μ m sterile filter. A working concentration of 100 μ M (unless stated otherwise) was selected.

Immunohistochemistry

Immunohistochemical staining was performed on serial sections of tissue multiblocks. Endogenous peroxidase was blocked in 1 % H₂O₂-methanol for 20 min at room temperature. Antigen retrieval was achieved by pressure-cooking the tissue samples in 10 mM citrate

buffer (pH= 6). After the slides cooled (> 10 min), they were incubated with primary antibody for 1 hour at room temperature (MT I/II – Dako, clone E9, 1:50; melan-A – Dako, clone A103, 1:50; DC-SIGN – R&D Systems, clone 120507, 1 µg/ml; CD68 – Dako, clone KP1, 1:100; CD163 – Novocastra, clone 10D6, 1:100; CD1a – Beckman Coulter, clone O10, 1:3). For visualisation of the reaction, the Envision/HRP detection system (Dako) and the VIP peroxidase substrate kit (Vectorlabs) were used. The slides were counterstained with methyl green. The stained tissue samples were digitalised using Pannoramic Viewer 1.15 (3DHISTECH Ltd.) and evaluated in blinded manner. MT I/II expression was evaluated semiquantitatively according to the intensity and extent of the immunoreactivity: score 0 (negative or weak diffuse staining, or strong positivity in < 10 % of the tumor cells) and score 1 (strong positivity in > 10 % of the tumor cells). We used the same criteria for assessing the MT I/II labelling of nevus cells as well. CD1a+, DC-SIGN+, CD68+, and CD163+ cells infiltrating or surrounding the tumor and MT I/II expressing cells within the tumor environment were evaluated according to their absence or presence.

Apoptosis assay

Cells were pre-treated with ZnCl₂ for 24 h and then exposed to UVB. Afterward, apoptosis was measured 24 h after UVB exposure. Harvested cells (5 x 10⁵) were washed in ice-cold PBS and stained with Annexin V and propidium iodide (PI) using the Vybrant apoptosis assay Kit (Invitrogen, Eugene, Oregon, USA) according to the manufacturer's protocol. The stained cells were analysed by flow cytometry (CyFlow® Space, Partec, Canterbury, United Kingdom) using UV/488 nm dual-excitation, measuring the fluorescence emission at 530 and 575 nm. Annexin V-PI- cells were identified as viable cells, Annexin V+PI- cells were identified as early apoptotic cells, and the sum of the Annexin V+PI+ and Annexin V-PI+ cells was identified as late apoptotic plus necrotic cells.

Cell proliferation

Cells were treated with 50 or 100 µM ZnCl₂ for 72 h, and cell proliferation was measured using the EZ4U assay (Biomedica, Vienna, Austria) according to the manufacturer's instructions. The optical density (OD) was determined at 450 nm using an Anthos 2020 microplate reader (Biochrom Ltd., Cambridge, UK).

CPD-ELISA

Cells were treated with ZnCl₂ for 24 h and/or UVB irradiated, and genomic DNA was purified 0, 1, 3, 6, and 24 h after UVB exposure using a QIAamp Blood Kit (Qiagen, Hilden, Germany). Ninety-six-well plates were coated with 0.003% protamine sulphate (Sigma-Aldrich, St. Louis, MO, USA) for 2 days at 37°C. To measure CPD lesions, genomic DNA was diluted to 0.2 µg/mL in PBS, denatured at 100°C for 10 min, and then chilled on ice for 15 min. The denatured DNA solution was transferred to plates pre-coated with protamine sulphate and dried overnight at 37°C. For blocking, the plates were washed four times with PBS-T (0.05% Tween-20 in PBS) and then incubated in 20% FBS for 30 min at 37°C. The wells were then washed four times with PBS-T and incubated with the primary CPD antibody (#TDM-2, Cosmo Bio Co. Ltd., Tokyo, Japan) at a 1:1000 dilution in PBS for 30 min at 37°C. The wells were then washed and incubated with the horseradish peroxidase (HRP)-conjugated anti-mouse IgG secondary antibody (BioRad, Hercules, CA, USA) diluted 1:3000 in PBS for 30 min at 37°C. After incubation, the cells were washed four times with PBS-T and then once with citrate-phosphate buffer (pH 5.0). Following buffer removal, the wells were incubated with a substrate solution (o-phenylene diamine (Sigma-Aldrich, St Louis, MO, USA), H₂O₂, and citrate-phosphate buffer (pH 5.0)) for 10 min at 37°C, at which time the enzyme reaction was stopped with 2 M H₂SO₄. The absorbance was measured using an Anthos 2020 microplate reader (Biochrom Ltd., Cambridge, UK) at 492 nm, and the OD values of the untreated cells were subtracted from those of the treated cells.

TaqMan Low-Density Array

Cells were treated with ZnCl₂ for 4 or 24 h, and relative gene expression was measured. In a parallel experiment, cells were pre-treated with ZnCl₂ for 24 h and then UVB irradiated. Six hours after exposure, total RNA was isolated using Trizol Reagent (MRC Inc., Cincinnati, Ohio, USA), and total RNA was quantified using a Nanodrop 2000 (Thermo Fisher Scientific, Wilmington, USA). cDNA was then synthesised using the High-Capacity cDNA Reverse Transcription kit (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's instructions. We designed Custom TaqMan Array (TLDA) Microfluidic Cards (384-well) by first selecting 91 genes based on their roles connected to zinc and the UVB response. The selected genes were involved in cell cycle progression, inflammation, apoptosis, DNA repair, and antioxidant defence.

Genes connected with zinc homeostasis and some with an identified metal regulatory element (MRE) in the promoter region were also added. The TLDA was performed using an Applied Biosystems 7900HT Real-Time PCR System according to the manufacturer's

protocol. cDNA (100 ng) in 100 μ L of 1X Taqman Gene Expression Master Mix (Applied Biosystems, Foster City, CA, USA) was loaded into each port of the TLDA plates, and each sample was examined in pairs. The PCR program used for amplification was as follows: 94°C for 1 min; 40 cycles of 94°C for 12 sec; and 60°C for 45 sec. The data were derived from three independently performed experiments and were analysed using SDS 2.1 software. ACTB, GAPDH, SDHA, and PGK1 were used for normalisation. Relative gene expression values were calculated using the 2-ddCt method.

Measurement of O₂^{•-} production

After 4 or 24 h of ZnCl₂ treatment or after 24 h of ZnCl₂ treatment combined with UVB, the production of O₂^{•-} was measured using a dihydroethidium (HE) assay (Sigma-Aldrich, St. Louis, MO, USA) 1, 4, 10, and 24 h after UVB exposure. A working solution of HE at a 2 μ M final concentration was added to the cells and then incubated for 30 min at 37°C. The cells were then harvested, and the fluorescence intensity was measured by flow cytometry with a FACSCalibur (BD Biosciences, San Jose, USA) to determine the fluorescence intensity at 450 nm, with 620 nm as a reference. The production of O₂^{•-} was expressed as the mean of HE-fluorescence intensity.

Detection of hydrogen peroxide

After 4, 10 or 24 h of ZnCl₂ treatment, hydrogen peroxide production was determined using an 10-acetyl-3,7-dihydroxyphenoxazine (Amplex red) assay. In this assay, a working solution of Amplex Red (Invitrogen, Eugene, Oregon, USA) at a 50- μ M final concentration and horseradish peroxidase (Sigma-Aldrich, St. Louis, MO, USA) at a 0.1 U/ml final concentration was added to the cells and then incubated for 20 min at room temperature. Fluorescence was subsequently read, with excitation at 530 nm and emission at 590 nm, using a Fluoroskan Ascent FL plate reader (Thermo Scientific, Vantaa, Finland). The MFI values of untreated cells were subtracted from those of the treated cells.

Western blotting

Cells were treated with ZnCl₂ for 4, 24, 48 or 72 h and lysed in RIPA buffer (Sigma-Aldrich, St. Louis, MO, USA). The protein concentration was then estimated using a BCA protein assay kit (Pierce Biotechnology, Rockford, USA). Proteins (20 μ g) were separated on 10 or 12% SDS-polyacrylamide gels and then transferred onto nitrocellulose membranes by electroblotting. The membranes were then blocked with 5% low-fat milk in TTBS (0.1%

Tween-20 in Tris-buffered saline) or in PBST (0.05% Tween-20 in PBS) and incubated overnight with primary antibody against heme oxygenase-1 (HMOX1) (#ab13248, Abcam Inc., Cambridge, USA) at 1:250 dilution or MTI/II (#ab12228, Abcam Inc., Cambridge, USA) at 1:1000 dilution in 5% milk at 4°C. The membranes were then washed and incubated with horseradish peroxidase-conjugated secondary antibody for 1 h at room temperature. After washing, the signals were visualised using ECL™ Prime Western Blotting Detection Reagent (GE Healthcare, Freiburg, Germany). B-actin (Cell Signaling Technology Inc., Danvers, MA, USA) was used for normalisation.

Immunofluorescence and confocal microscopy

The HaCaT cells were plated on glass coverslips, treated with ZnCl₂ for 24 h or/and then UVB irradiated. 3 h after UVB, cells were fixed (20 min) with 4% paraformaldehyde. They were permeabilised with 1% TritonX-100 (10 min), blocked with 20% FBS, and then incubated overnight with primary antibodies against all MT I/II (#ab12228, Abcam Inc., Cambridge, USA) at a 1:50-dilution in PBS at 4°C. After washing with PBS, the preparations were incubated with a goat anti-mouse IgG Alexa Fluor 555 antibody for 2 h in PBS. Coverslips were mounted using an anti-fade reagent (Vector Laboratories Inc., Burlingame, CA, USA) and analysed using an Olympus FV1000S confocal microscope (Olympus Co. Tokyo, Japan) using the 60x oil-immersion objective (NA: 1.3) (Tamás Juhász). For excitation, a laser of 543 nm was used. The average pixel time was 4 μs. The Z image series of 1 μm optical thickness were recorded in sequential scan mode. Confocal settings were identical for all scans and remained constant during imaging.

Statistical Analysis

The data were statistically analysed using t-test, the Mann-Whitney U-test, Pearson χ^2 -test, Fisher's exact test and Spearman's Rho correlation (GraphPad Prism version 5.03, GraphPAD Software Inc., San Diego, CA, USA; SPSS for Windows, version 19.0, SPSS Inc., Chicago, IL, USA). Correlations were defined as significant if $p < 0.05$.

RESULTS

Clinicopathological data

We studied 46 primary CMMs after dividing them into 2 groups, one nonmetastatic and the other exhibiting hematogenous metastases. The patients without metastases were followed up for 8-13 years (average 10 years). Comparing the clinicopathological parameters of the tumors with different metastatic potential, we found that the average Breslow tumor thickness was significantly higher in the metastatic group ($p= 0.001$) and that there were more metastatic cases among male patients (2.14:1) and fewer among females (1:2) ($p= 0.020$). In the metastatic group, more tumors were localised to the trunk and to the head and neck region than in the nonmetastatic group (78.3 % vs. 56.5 %), and more CMMs were of the nodular subtype (43.5 % vs. 34.8 %). However, these differences were not significant. As expected, the proportion of exulcerated CMMs was higher among the metastatic tumors (56.5 % vs. 13 %). There was no significant difference between the groups in patient age ($p= 0.577$) or in the Clark invasion level ($p= 0.584$).

Tumoral MT I/II (MTt I/II) expression in primary CMM

The groups of nonmetastatic and metastatic primary CMM were compared with respect to the intensity, extent and distribution of the MTt I/II staining. The basal keratinocytes, the proliferating epithelium of the follicular bulb and the ductal epithelium of the eccrine and apocrine glands served as positive controls for MT I/II immunohistochemical staining. The cytoplasm and the nucleus of the tumor cells were positively labelled. To determine the suitability of TMA for the evaluation of MTt I/II expression in CMM, tissue samples were cut from the epidermal, dermal and central regions of each tumor (at least one section from each area). Although most metastatic CMMs exhibited diffuse staining of all tumor parts, we found that the dermal/central compartments of the melanomas were MTt I/II-labelled in each positive case. In our preliminary short pilot study, we detected the same immunoreactivity pattern in whole sections. In several primary tumor samples, single tumor cells at a distance from the main tumor mass could be observed with intensive MTt I/II staining. Considering the intensity and extent of MTt I/II labelling, the difference between nonmetastatic and metastatic primary tumors was significant when we defined tumors with less than 10 % of tumor cells positively stained or with diffuse pale staining (score 0) to be negative and tumors with more than 10 % of tumor cells labelled (score 1) to be positive ($p= 0.018$).

The Breslow tumor thickness correlated with the risk of metastasis in our CMM cases, but it did not correlate with the MTt I/II expression.

The median tumor thicknesses of the MTt I/II-positive (n= 22) and -negative (n= 24) CMMs were 2.8 mm and 2.6 mm, respectively. The median tumor thicknesses of metastatic MTt I/II-positive and -negative CMMs (3.5 mm vs. 3.15 mm), as well as the thicknesses of nonmetastatic MTt I/II-positive and -negative primary tumors (1.5 mm vs. 1.23 mm), were also similar. Interestingly, the MTt I/II score correlated with age (R= 0.349, p= 0.017). There was no correlation between gender and the MTt I/II score.

MT I/II expression in nevi (MTn I/II)

Benign pigment cell nevi (n= 6; male:female= 1:5; mean age= 48 (35-63 years); 4 compound type, 1 junctional nevus and 1 dermal-type nevus) were also investigated for MTn I+II expression. All the nevi showed MTn I+II expression (score 1), but the labelling pattern was different from that observed in primary CMM. Namely, nevus cells were stained immediately below the epidermis, whereas MTt I+II-positive melanoma cell nests were localised to the central/dermal part of the tumor. In addition, we observed pronounced nuclear MTn I+II staining in the nevi, but the CMM cells showed intensive cytoplasmic and nuclear immunoreactivity.

Peritumoral MT I/II (MTp I/II) expression

MT I+II expression of the basal layer of the epidermis is well described and served as a positive internal control in our immunohistochemical study. In normal human skin samples, we observed MT I/II expression in dermal cells, which were characterised by dendritic and spindle cell morphologies and by their association with perivascular lymphocytes. MTp I/II+ cells were also observed surrounding the tumor cell nests; however, their presence did not correlate with the metastatic potential of the primary CMMs (p= 0.555).

Tumor-infiltrating and peritumoral CD68, CD163, DC-SIGN and CD1a expression

We determined the presence of CD68+, CD163+, DC-SIGN+ and CD1a+ macrophages/dendritic cells separately within the tumor and around the tumor cell nests. We observed significant differences between the groups of metastatic and nonmetastatic CMM with respect to the presence of tumor-infiltrating CD68+ and CD163+ macrophages and peritumoral CD1a+ DCs.

Significantly more metastatic CMM cases were associated with the presence of CD68+ ($p= 0.001$) and CD163+ ($p< 0.001$) cells infiltrating the tumor, but significantly fewer metastatic primaries were associated with the presence of CD1a+ cells ($p= 0.003$) surrounding the tumor. Epidermal CD1a+ Langerhans cells were observed in each tissue sample within the epidermis without any obvious difference between the groups. We were then interested whether there is any correlation between the presence of immune cells and CMM MTt I/II positivity. Some overlap regarding the localisation/distribution of DC-SIGN+ cells and MTp I/II-labelled peritumoral cells could be seen in serial sections, but this observation was not significant. However, we determined a significant linear correlation between tumor-infiltrating CD68+ macrophages and the MTt I/II expression of the melanoma cells ($p= 0.003$).

Zn (II) increased cell proliferation

To examine the effect of Zn (II) exposure on cell proliferation, HaCaT cells were treated with Zn (II) at concentrations of 50 and 100 μM in growth medium containing 10% FBS, and the effects were measured using an EZ4U assay. We found that 72-h treatment with 100 μM Zn (II) resulted in significantly increased cell proliferation (1.3-fold; $p=0.045$).

Zn (II) exposure impacted the expression of genes involved in zinc homeostasis, antioxidant defence, cell viability and inflammation in HaCaT cells

Then, we identified genes that showed altered expression in response to 100 μM Zn (II)-containing medium using TLDA analysis. We detected six significantly up-regulated genes with relative expression levels of at least 1.5-fold in Zn (II)-treated cells compared to the untreated controls. Isoforms of MT showed different transcriptional activity at base level. Gene expression of MT2A was the highest, and it was followed by MT1X, MT1E and MT1F. The transcriptional change of the isoforms to ZnCl₂ exposure varied from the sequence of the basic expression levels. We detected induction of MT gene expression at 4 h of Zn (II) treatment and the induction of MT1F, MT1X and MT1E was greater than the transcriptional change of MT2A. MT1F gene showed the highest overexpression at both time points (40.86-fold at 4 h; 12.92-fold at 24 h), and MT1X and HMOX1 were expressed more than 10-fold higher in treated cells after 4 h of Zn (II) exposure. The expression levels of MT1E, MT2A and SLC30A1 also showed at least a 2-fold significant difference between treated and untreated cells at both treatment times. The notch homolog 1, translocation-associated (*Drosophila*) (NOTCH1) gene was slightly up-regulated (1.47-fold), and we detected slight

but significant suppression of IL8, prostaglandin-endoperoxide synthase 2 (PTGS2), and cytochrome P450 1B1 (CYP1B1) mRNA expression (0.63-fold, 0.68-fold, and 0.63-fold, respectively) after 4 h of Zn (II) treatment. Specific commercial antibodies against MT protein isoforms are not currently available, and the composite of MTI/II can only be investigated at the protein level. To validate our mRNA data we investigated the protein expression level of MTI/II by western blot, and we observed MTI/II induction compared to control with a maximum at 48 h after Zn (II) treatment.

Zn (II) exposure impacted the expression of HMOX1 and induced significant O₂^{•-} production

We also measured the protein expression of HMOX1 after 4 and 24 h of Zn (II) exposure. Western blot analysis demonstrated that 4-h Zn (II) treatment induced the production of a 34-kDa HMOX1 protein. On the other hand, the induction of the antioxidant defence protein HMOX1 upon Zn (II) exposure prompted us to examine whether Zn (II) treatment can induce reactive oxygen species. We measured the generation of O₂^{•-} in response to Zn (II) exposure in cells using a flow cytometry-based HE assay. Our data showed significant production of O₂^{•-} after 4 h of Zn (II) treatment compared to the control (p=0.006). To further confirm our data, we investigated O₂^{•-} dismutation by measuring the production of H₂O₂, and we found extracellular diffusion of H₂O₂ to the growth medium 24 h after Zn (II) treatment (p=0.019).

Zn (II) pre-exposure decreased the amount of CPDs but enhanced UVB-induced O₂^{•-} generation

To shed light on the mechanism of the zinc-mediated component of the UVB response, we characterised UVB-induced O₂^{•-} generation and CPD formation after 24-h Zn (II) pre-treatment. We examined the level of O₂^{•-} in Zn (II) pre-treated, UVB-irradiated cells and in cells only exposed to UVB. We observed an increase in O₂^{•-} generation, with a significant plateau 10 h post-UVB irradiation compared to the control (p<0.001), and we detected a time-dependent decrease in the amount of CPDs after UVB irradiation. Zn (II) pre-treatment changed both processes that were induced by UVB. Our results showed that significantly less CPDs (by 19.64%) were detected 3 h after UVB irradiation in cells pre-treated with Zn (II) for 24 h compared to cells exposed only to UVB (p=0.0082). However, at 10 h post-UVB exposure, significant enhancement of superoxide generation was observed

when comparing Zn (II) pre-treated, UVB-irradiated cells to cells only treated with UVB irradiation ($p < 0.001$).

Zn (II) pre-exposure altered the type of cell death after UVB irradiation, without influencing the overall survival

To investigate the effect of reduced levels of CPDs but elevated $O_2^{\bullet-}$ levels after Zn (II) pre-treatment, flow cytometric apoptosis and EZ4U cell viability assays were used. We found that the cell survival did not change between Zn (II)-treated and control cells 24 h after UVB irradiation.

Regarding control cells, the proportion of viable cells was 88.69%, and in UVB irradiated cells this ratio decreased to 48.2%. The proportion of viable cells during Zn (II) exposure was 91.71%, and similar decrease was observed in Zn (II) pre-treated, UVB irradiated cells (to 50.1%). On the other hand, Zn (II) pre-treatment significantly decreased the proportion of early apoptotic cells (Annexin V+PI-; by 5.94% or 0.57-fold, $p = 0.0286$) and significantly increased the population of late apoptotic plus necrotic cells (Annexin V+PI+ and Annexin V-PI+; by 4.61 % or 1.12-fold, $p = 0.0026$).

Zn (II) caused a transcriptional shift in the UVB-modulated expression of genes

To determine the molecular mechanism behind the cellular response to the combined effects of Zn (II) and UVB exposure, we performed a TaqMan Low-Density Array analysis to characterise the gene expression changes in HaCaT cells pre-treated with Zn (II). We identified 39 differentially expressed genes in cells only submitted to UVB irradiation 6 h after exposure compared to non-irradiated control cells. Twelve genes were expressed more than 2-fold higher in UVB-irradiated cells than in control cells, and TNF, PTGS2, and IL-8 showed the highest overexpression (>10-fold). We identified 27 genes that were down-regulated in UVB-irradiated cells compared to the control cells; MT1E was the most repressed (0.12-fold). Furthermore, MTF1 levels were significantly decreased compared to the control (0.32-fold). We observed a modest modulation in gene expression when comparing Zn (II) pre-treated, UVB-irradiated cells to those that were only UVB-irradiated. Zn (II) pre-exposure mainly reduced UVB-induced homeodomain interacting protein kinase 2 (HIPK2) mRNA suppression and caused little modification of polymerase (DNA directed), beta (POLB) and RAD51 homolog (RecA homolog, E. coli) (*S. cerevisiae*) (RAD51) mRNA levels, whose relative gene expression levels were repressed following UVB irradiation (<0.75-fold) but remained unchanged (0.76-1.5-fold change) when pre-treated with Zn (II).

Cellular distribution of MTs in Zn (II) pre-treated cells and after UVB irradiation

We investigated the cellular distribution of MTI/II by confocal microscopy. MTs were present mainly in the nucleus of the untreated cells and we found that Zn (II) induced immunostaining of MTs in the cytosol. UVB treatment changed the picture of Zn (II) pre-exposure, partial translocation of MTs to the nucleus was observed 3 h after UVB irradiation.

DISCUSSION

Zinc is related to a number of physiological functions, and zinc deficiency has enormous consequences as described in skin diseases. The use of zinc-containing topical therapeutics is widespread due to its clear benefit on cutaneous regeneration and on various inflammatory skin conditions. However the events involved in the regulation of zinc homeostasis are complex and incompletely understood. MT has a crucial role in the maintenance of the balance of intracellular labile zinc level in cells. It has a sequestering function, which serve zinc for cell biological processes. It has been demonstrated that it can protect normal cells from damage by protecting against oxidative stress, enhancing tissue repair and modulating the immune response and MT knockout is lethal. However, MT overexpression can also contribute to a more aggressive phenotype and therapy resistance in cancer cells, resulting in a worse prognosis. The role of MT in metastasis formation remains to be confirmed, and experimental evidence for its oncogenic role is still lacking. On the other hand, UVB, the main harmful environmental factor in skin, and crucial risk factor of skin photocarcinogenesis, induces intracellular zinc release in keratinocytes, suggesting a functional role of zinc in the UV stress response. Investigating the role of zinc homeostasis under physiological circumstances and under stress conditions can give an insight into the role of zinc in the course of skin diseases, and can add new therapeutic possibilities.

MT as a promising biomarker in CMM prognosis

In the present study, a statistically significant difference was demonstrated between nonmetastatic and metastatic CMM regarding their MTt I/II expression, where positive immunostaining was defined as intensive MTt I/II labelling of more than 10 % of the tumor cells. Both the cytoplasm and the nucleus of the cancer cells showed intensive staining. The whole tumor or at least the dermal part of the tumor was positively labeled; therefore, the TMA appears to be appropriate way to investigate MTt I/II expression in CMM. It is important, because the demand on tissue sample for genetic testing determining the therapy is increasing, but also for more precise estimation of prognosis, and both procedure requires eligible amount of tumor tissue.

We found that there was no significant correlation between the Breslow tumor thickness and the MTt I/II score ($R= 0.102$, $p= 0.501$), suggesting that the two parameters are independent prognostic factors.

For the immunological characterization of our CMM samples we used dendritic cell markers such as, CD1a and DC-SIGN, macrophage markers, such as CD68 and CD163 and T-cell markers, such as CD45RO and CD8. We have demonstrated a linear correlation between the metastasis risk of primary CMM and the presence of tumor-infiltrating CD68+ and CD163+ macrophages as well as a correlation between the presence of peritumoral CD1a+ cells and a significantly lower risk for CMM metastasis. The tumor microenvironment may play an important role in melanoma progression, and it has been previously demonstrated that the presence of infiltrating macrophages within melanomas is associated with poor prognosis; these fail to induce cytotoxic T-cell response against cancer cells, and are related to tissue remodeling and neoangiogenesis and we found that the presence of CD8 positive cytotoxic T-cells was inversely correlated with the progression of CMM. The immunostaining for all of these markers were dependent on the tumor thickness. We found that the MTt I/II expression of the melanoma cells was correlated with the presence of CD68+ macrophages infiltrating the tumor, which has not been previously reported. Macrophage infiltration of primary tumors is regulated by cytokines, growth factors and enzymes released from the tumor. Tumor derived factors can also control the function of the tumor-associated macrophages. Activation of M1 macrophages causes production of pro-inflammatory cytokines and induction of type 1 T-cell response capable of killing tumor cells (arrested tumor growth and invasion). However, during tumor progression M2-type macrophages are induced, which are thought to be the main tumor-associated macrophages. M2-type macrophages suppress the inflammatory response, impair the antigen presentation and type 1 immune responses, induce tissue remodeling, neoangiogenesis (promoted tumor growth and invasion). Inflammatory stimuli can upregulate the expression of MT I/II. It has been demonstrated that in animal model of brain injury, during stress conditions, MT I/II is produced mainly by reactive astrocytes and the elevation of MT expression level in tissue may act on the inflammatory microenvironment suppressing IL-6 and TNF- α production of macrophages, decreasing apoptotic cell death in neurons and oligodendrocytes, and enhancing tissue repair. Extracellular MT released from cells has been shown to operate as a leukocyte chemotactic factor and to interact with the plasma membrane of immune cells (lymphocytes, macrophages) resulting in a change in the immune functioning (e.g., it decreases the differentiation of immature T-cells to the effector cytotoxic T-lymphocyte stage). These findings suggest that MT might play a role in modulation of immune microenvironment and support that MT over-expression in melanoma might be associated with the macrophage infiltration and tumor promoting microenvironment. As it is certified by a recently published review article, the results of our research work highly

contributed to confirm the prognostic value of MT I/II expression level in CMM. Moreover, another recent study has demonstrated low MT III expression in normal skin, but significant induction of MT III in SCC justifying the importance of further investigations on the role of MT proteins in skin cancer development and progression.

MT expression in human keratinocytes

MT is a small protein with 6-7 kDa molecular weight and high cysteine content. It has important role in intracellular zinc sequestration and through this, it is involved in the zinc dependent cell biological processes. Bozym et al. investigated the effect of cell culture medium on the intracellular Zn (II) concentration and found that both the type of cell and the medium influence the intracellular free Zn (II) level during exposure to a certain concentration of Zn (II) and that the toxicity of supplemented Zn (II) must be carefully investigated. Previous experiments have shown that the exposure of HaCaT keratinocytes to 100 μ M ZnCl₂ was non-toxic to the skin cells, and we have not found this concentration to be toxic in our experimental conditions. Based on the calculations of Bozym et al., 100 μ M Zn (II) treatment leads to an approximate 100-nM increase of intracellular Zn (II). To get a deeper insight into cell biological functions of zinc in human HaCaT keratinocytes, we studied the transcriptional response in cells exposed to nontoxic concentration of ZnCl₂. We also investigated the mRNA expression of MT isoforms. More than 10 functional MT isoforms are present in humans and are classified into four groups; we analysed the mRNA expression of nine of the MT isoforms from the MT I/II groups in keratinocytes in our experiments. At a baseline level we detected expression of 4 MT-isoforms in HaCaT cells (MT2A>MT1X>MT1E>MT1F). There are only a few studies concerning MT expression in keratinocytes, Lim et al. also have found MT2A to be the most abundant MT-isoform. MT expression seems to be oscillating during cell cycle and nuclear translocation of MT can be observed during early S-phase. The fact that isoforms of MT1 are encoded by different genes and results of the few studies demonstrating prognostically relevant expressional change of distinct MT1 isoforms in cancers together with our results suggest that the isoforms have specific regulatory functions and it is still little known so far. Zn (II) treatment significantly increased the mRNA expression of all four MT isoforms and SLC30A1. Transcriptional change of MT1F, 1X and 1E was superior to the change of MT2A showing that expression of these are more sensitive to change of intracellular zinc concentration. Western blot analysis demonstrated that Zn (II) treatment induced the production of MTI/II protein in a time-dependent manner. In addition, we investigated the cellular localisation of MTs and found

that MTs were present mostly in the nuclei of control cells, suggesting a function in defence of genome and/or cell growth. It is consistent with our immunohistochemical results demonstrating nuclear MT immunolabelling in nevi. However after 24 h of treatment with Zn (II), we detected high amounts of MTs in the cytoplasm of the cells, and little or no fluorescence staining was detected in the nucleus. The variability of the localisation of MTs upon zinc exposure may reflect the expression of distinct MT isoforms.

The effects of zinc on the functions of human HaCaT keratinocytes

In the present study we found a significant proliferation advantage upon 100 μ M ZnCl₂ exposure, in accordance with a previous study. Although the effects of zinc on intracellular signalling events are not completely known, a positive impact of Zn (II) on cell proliferation might be attributed, at least in part, to the inhibition of phosphatases, leading to augmented protein tyrosine phosphorylation. The proliferation advantage by Zn (II) treatment can also be a consequence of increased calcium uptake or up-regulated gene expression (e.g., NOTCH1). In addition, as mentioned above, in aged skin, which is characterised by decreased epidermal renewal, the expression of MT is lower, while the proliferative layer of the epidermis shows high MT expression. Moreover, our finding that MT is overexpressed in CMM with poor prognosis suggests a role of MT in hyperproliferative capacity of cells.

The mechanism of action of zinc impact on cell signalling is not well understood; herein, we found that zinc might affect ROS-sensitive signalling pathways. This is the first report demonstrating high expression of HMOX1 in cultured human keratinocytes upon nontoxic Zn (II) exposure. The HMOX1 gene encodes an important member of the phase-II enzymes, which have cytoprotective roles in ROS scavenging. Furthermore, HMOX1 was shown to prevent cell death and to have a role in the regulation of inflammation. Nontoxic generation of O₂^{•-} with subsequent H₂O₂ release was detected during nontoxic Zn (II) exposure and could explain the HMOX1 expression. Although Zn (II) itself is reported to be an inducer of oxidative stress by promoting mitochondrial and extra-mitochondrial production of ROS, the generation of ROS at a nontoxic level upon Zn (II) exposure/release has not been described. Nevertheless, O₂^{•-}-producing NAD(P)H oxidases are activated in response to growth factors and oncogenes, and enhanced cell proliferation was observed upon ROS-inducing, low-dose photodynamic treatment. Therefore, simultaneous increases in cell proliferation and ROS levels are not conflicting. In addition, NADPH oxidase mediated the induction of nuclear factor-kappa B (NF κ B) and HMOX1 in human colon cancer cells without inducing cell death. Furthermore, the immune response can be redox-regulated, and we have observed down-

regulation of some pro-inflammatory mediators, such as IL8 and PTGS2, which are partly regulated by redox-sensitive transcription factors (e.g., AP-1).

Biological role of zinc in UVB induced stress response in human skin HaCaT keratinocyte cell model

UVB exposure is the primary risk factor in skin-tumor development. A role of zinc in the mechanism of UVB-induced cell death has already been proposed. Stork et al. described that the elevation of intracellular zinc levels after UVB irradiation is proportional to the fraction of dying or dead cells, and they concluded that UVB-induced zinc release may be an important step in UVB-induced cell death pathways. The consequences of UVB-induced direct (primarily CPDs) and indirect (free radical generation) DNA damage are cell cycle arrest, DNA repair, and the induction of cell death pathways causing sunburn, inflammation, immunosuppression, and melanogenesis in the skin. We measured both the CPD level and superoxide generation. Due to DNA repair processes, the amount of CPDs was decreased with time, and cells with unrepaired DNA damage were eliminated by apoptosis. Similar to the results of Saito et al., pre-treatment of cells with Zn (II) for 24 h was not sufficient to improve cell survival, although the level of induced CPD was lower in these cells 3 h after UVB irradiation. We assume that MT could exert some effect on CPD formation because partial translocation of MTs to the nucleus was observed 3 h after UVB irradiation. Nevertheless, when further analysing cell death at 24 h post-UVB irradiation, when UVB-induced apoptosis is maximal, we found that the fraction of early apoptotic cells decreased, while the fraction of late apoptotic plus necrotic cells increased in Zn (II) pre-treated cells. In the context of our results, the elevation of intracellular zinc levels after UVB irradiation that was described by Stork et al. was proportional to the fraction of dying or dead cells. When investigating the course of ROS production, the increase in superoxide production upon UVB treatment occurred at a maximum of 10 h, but unexpectedly, this was augmented by Zn (II) pre-exposure. It has recently been described that a vicious circle of ROS-induced zinc release and zinc-driven mitochondrial ROS production is an important neuronal cell death mechanism. On the other hand, it has been reported that the induction of a type of cell death other than apoptosis in cancer cells might increase the immunogenic potential of cell death. Whether a change in the death process upon Zn (II) pre-exposure can affect the immune response to UV-damaged cells, which would impact the development of various pathologies, e.g., autoimmunity or cancers, must be further studied.

The mRNA levels of MT1E and MTF1 were strongly suppressed by UVB at 6 h after irradiation. Epigenetic down-regulation of MT1E has been demonstrated in malignant melanoma, suggesting it exhibits tumor-suppressor function. Only a small fraction of genes showed altered expression upon zinc pre-treatment at 6 h post-UVB irradiation. Interestingly, expression of HIPK2, which is involved in regulatory phosphorylation of key molecules such as p53, showed the biggest change in gene expression after combined zinc and UVB exposure. Furthermore, the generated ROS can modulate the selective transactivation of p53 target genes.

SUMMARY

The purpose of our study was to increase our knowledge on pathogenesis of skin disorders regarding the role of zinc homeostasis and UVB-induced DNA damage response.

Comparison of the MT I/II protein expression of primary CMM tissue samples without metastases to samples with haematogenous metastases:

We found statistically significant difference between nonmetastatic and metastatic CMM regarding their MT I/II expression. The MT I/II expression did not correlate with the Breslow tumor thickness i.e. it seems to be an independent prognostic factor. In addition, this was the first study to investigate correlation between MT I/II expression and composition of tumor immune microenvironment in CMM. We found that MT I/II expression of the melanoma cells was correlated with the presence of CD68⁺ macrophages infiltrating the tumor.

Investigation of the effect of non-toxic Zn (II) exposure on human epidermal keratinocytes under physiological circumstances and stress conditions:

In our study non-toxic elevation in the concentration of extracellular zinc facilitated cell proliferation. This is the first report that this concentration of zinc increased HMOX1 expression and caused non-toxic generation of superoxide in skin keratinocytes. Furthermore, regarding the effects on the UVB-induced toxicity, we could show that although the level of cyclobutane pyrimidine dimers in the keratinocytes pre-treated with zinc was reduced at early time point after UVB irradiation, significantly enhanced superoxide generation was observed later after UVB exposure in the zinc pre-exposed cells. The overall survival was unaffected; however, there was a decrease in the percentage of early apoptotic cells and an increase in the percentage of late apoptotic plus necrotic cells.

In conclusion, by these observations we can assume that MT, and through this, zinc dyshomeostasis might contribute to melanoma progression. Regarding non-toxic zinc exposure, the observed changes in ROS might be attributed to the molecular effects of Zn (II) interactions with cysteinyl thiols, which alters protein functionality and thereby their reactivity and participation in redox reactions. It can affect signal transduction pathways, which further may influence the behaviour of the cell upon stress conditions. Our observations affect our thinking about the pathogenesis of skin disorders and pay attention to further examine the role of the zinc homeostasis in skin.

PUBLICATIONS



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List of publications related to the dissertation

1. **Emri, E.**, Mikó, E., Bai, P., Boros, G., Nagy, G., Rózsa, D., Juhász, T., Hegedűs, C., Horkay, I., Remenyik, É., Emri, G.: Effects of non-toxic zinc exposure on human epidermal keratinocytes.
Metallomics. 7 (3), 499-507, 2015.
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J. Eur. Acad. Dermatol. Venereol. 27 (3), e320-e327, 2013.
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List of other publications

3. Boros, G., Mikó, E., Muramatsu, H., Weissman, D., **Emri, E.**, van der Horst, G.T.J., Szegedi, A., Horkay, I., Emri, G., Karikó, K., Remenyik, É.: Identification of Cyclobutane Pyrimidine Dimer-Responsive Genes Using UVB-Irradiated Human Keratinocytes Transfected with In Vitro-Synthesized Photolyase mRNA.
PLoS One. 10 (6), e0131141-, 2015.
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4. Boros, G., Mikó, E., Muramatsu, H., Weissman, D., **Emri, E.**, Rózsa, D., Nagy, G., Juhász, A., Juhász, I., van der Horst, G., Horkay, I., Remenyik, É., Karikó, K., Emri, G.: Transfection of pseudouridine-modified mRNA encoding CPD-photolyase leads to repair of DNA damage in human keratinocytes: A new approach with future therapeutic potential.
J. Photochem. Photobiol. B. 129, 93-99, 2013.
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International oral presentations related to the dissertation:

E Emri: Correlation among metallothionein expression, intratumoral macrophage infiltration and the risk of metastasis in human cutaneous malignant melanoma. 44th ESDR Annual ESDR Meeting, Copenhagen, Denmark, 10-13 September 2014 *oral presentation*

E Emri, E A Janka, G Boros, L Beke, Cs Hegedus, B Nagy, G Mehes, E Remenyik, G Emri: Correlation among anti-tumor T cell response and the redox homeostasis in human cutaneous malignant melanoma. 44th ESDR Annual ESDR Meeting, Copenhagen, Denmark, 2014; *J Invest Dermatol* 134: S90-S97 *poster presentation* DOI:10.1038/jid.2014.350

E Emri, E Miko, E Remenyik, G Emri: Zinc chloride exposure modulates cytokine expression and induces heme oxygenase-1 in human keratinocytes. International Investigative Dermatology Edinburgh, Scotland 2013; *J Invest Dermatol* 133: S191-S208 *poster presentation* DOI:10.1038/jid.2013.103

E Emri, G Emri, K Egervari, I Veres, G Mehes, E Remenyik: Prognostic role of CD68, CD163 and CD1a expression in human primary cutaneous malignant melanoma. 75th Anniversary of Albert Szent-györgyi's Nobel Prize Award, 22-25 March, 2012. *oral presentation*

E Emri, E Miko, G Nagy, G Boros, D Rozsa, G Mocsai, G Emri, E Remenyik: Modulating effect of zinc on cutaneous UVB-response. 42th ESDR Annual ESDR Meeting, Venice, Italy, 2012; *J Invest Dermatol* 132: S118-S123 *poster presentation* DOI:10.1038/jid.2012.306

E Emri, G Emri, K Egervari, L Beke, T Varvolgyi, I Veres, G Mehes, I Horkay, E Remenyik: Immunohistochemical characterization of metallothionein expression in primary cutaneous malignant melanoma. 41th ESDR Annual ESDR Meeting, Barcelona, Spain, 2011; *J Invest Dermatol* 131: S1-S115 *poster presentation* DOI:10.1038/jid.2011.214 79

G Emri, T Varvolgyi, A Begany, Z Hargitay, L Toth, E Emri, E Remenyik: Immunohistochemical markers as prognostic factors in primary cutaneous malignant melanoma. 39th Annual ESDR Meeting, Budapest, Hungary, 2009; *J Invest Dermatol* 129: S1-S103 *poster presentation* DOI:10.1038/jid.2009.232

National oral presentations related to the dissertation:

Emri E, Janka E A, Boros G, Beke L, Hegedűs Cs, Méhes G, Remenyik É, Emri G: A tumor-ellenes T-sejt válasz és a redox homeosztázis közötti összefüggés kután melanoma malignumban. Magyar Dermatológiai társulat 87. nagygyűlése, Budapest, Hungary; 27 November 2014. *oral presentation*

Emri E, Miko E, Boros G, Nagy G, Bai P, Mócsai G, Rózsa D, Hegedűs Cs, Remenyik É, Emri G: A cink homeosztázis befolyásolja a citokin-expressziót és hem-oxigenázt indukál humán keratinocitákban. Magyar Dermatológiai társulat 86. nagygyűlése, Budapest, Hungary; 12-14 December 2013. *oral presentation*

Emri E, Miko E, Nagy G, Boros G, Rózsa D, Mócsai G, Remenyik É, Emri G: A cink UVB sugárzás utáni sejtválaszra gyakorolt hatása keratinocitákban. Magyar Dermatológiai társulat 85. nagygyűlése, Budapest, Hungary; 6-8 December 2012. *oral presentation*

Emri E, Emri G, Egervári K, Beke L, Rózsa D, Miko E, Boros G, Nagy G, Várvölgyi T, Veress I, Méhes G, Remenyik É: A metallothionein-expresszió melanómában: tumorbiológiával és immunválasszal összefüggést mutató független biomarker. Magyar Dermatológiai társulat 84. nagygyűlése, Budapest, Hungary; 8-10 December 2011. *oral presentation*

Other presentations:

G Boros, E Miko, H Muramatsu, D Weissman, **E Emri**, G van der Horst, I Horkay, K Karikó, G Emri and E Remenyik: Effect of cyclobutane pyrimidine dimer on gene expression is mediated by activation of c-Jun kinase in UVB irradiated human keratinocytes. 44th ESDR Annual ESDR Meeting, Copenhagen, Denmark, 2014; *J Invest Dermatol* 134: S83-S89 *poster presentation* **DOI:10.1038/jid.2014.349**

Cs Hegedűs, G Boros, **E Emri**, E Mikó, K Karikó, G Emri, P Bai, É Remenyik: The implication of mitochondria in the UVB-driven pathways, 44th Annual ESDR Meeting Copenhagen, Denmark, 2014; *J Invest Dermatol* 134: S83-S89 *poster presentation* **DOI:10.1038/jid.2014.349**

G Boros, E Miko, **E Emri**, G van der Horst, H Muramatsu, D Weissman, I Horkay, G Emri, K Kariko, E Remenyik. 43rd Annual ESDR Meeting. Edinburgh, Scotland, 2013; *J of Invest Dermatol* 133: S209–S221 *poster presentation* **DOI:10.1038/jid.2013.104**

G Boros, E Miko, **E Emri**, G van der Horst, H Muramatsu, D Weissman, I Horkay, G Emri, K Kariko, E Remenyik: CPD-dependent gene expression changes in photolyase mRNA-transfected human keratinocytes irradiated by UVB. 42th ESDR Annual ESDR Meeting, Venice, Italy, 2012; *J Invest Dermatol* 132: S118-S123 *poster presentation* **DOI:10.1038/jid.2012.306**

G Boros, D Rozsa, **E Emri**, G Nagy, E Miko, A Juhasz, I Juhasz, G van der Horst, H Muramatsu, D Weissman, K Kariko, G Emri, E Remenyik, I Horkay: Enhanced repair of UVB-induced DNA lesions in photolyase mRNA-transfected keratinocytes. 41th ESDR Annual ESDR Meeting, Barcelona, Spain, 2011; *J Invest Dermatol* 131: S1-S115 *poster presentation* **DOI:10.1038/jid.2011.214**

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