The role of ultraviolet radiation in experimental and clinical dermatology

Doctoral theses

Nóra Gyöngyösi MD

Doctoral School of Clinical Medicine
Semmelweis University

Supervisor: Norbert M. Wikonkál, MD, Ph.D, D.Sc

Consultant: Márta Marschalkó, MD, Ph.D

Official reviewers: Éva Margittai, MD, Ph.D
Zsuzsanna Lengyel, MD, Ph.D

Head of the Complex Examination Committee:
Chair: András Arató, MD, Ph.D, D.Sc
Members: Kálmán Tory, MD, Ph.D
László Szabó, MD, Ph.D

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1. Introduction

The therapeutic use of ultraviolet radiation goes back for more than a century. The biological effects of UV subsets vary greatly because each part of the UV spectrum has a different way to damage the DNA and an ability to penetrate the skin. However, extreme UV exposure to the skin has many harmful dermatological implications, so it is expected to achieve the optimal benefit / risk ratio in phototherapy, i.e. to deliver the most necessary UV dose to produce the greatest therapeutic effect. The first step in phototherapy is to determine the minimum erythema dose, i.e., MED, which is the starting point for phototherapy in the clinic. It is not only used in phototherapy, but it is also suitable for diagnosing photodermatoses, studying the photosensitizing effects of various drugs, or studying the efficacy of sunscreens. In photobiological experiments, this terminology is also used in mice, although the reaction of mouse skin to UV radiation greatly differs from that seen in humans, not to mention the fact that various mouse strains display diverse skin changes. In mice, a single high-dose UV exposure is associated not only with erythema but also with an edema of varying degree, which is in all cases, a significant factor of the UV reaction.

Various types of phototherapies are being used in the treatment of inflammatory skin diseases, however, the list of diseases that respond well to phototherapy include also oncological diseases, such as cutaneous T-cell lymphomas. Lymphomatoid papulosis (LyP) is the second most common disease among cutaneous lymphomas, and because the list of therapeutic options is rather limited, so light therapy is of great importance. The biggest benefit is that it is primarily directed for the skin with virtually

no systemic side effects. The classification of Lyp is regularly updated with the development of histological features and the development of molecular diagnostics, so we have even more information about rare variants of LyP. So far, CD8 positivity has only been associated with LyP type D, however, there are reports of individual cases of CD8 positivity in other LyP types as well.

2. Aims

In our work, we aimed to examine the skin of three mouse strains of different genotypes frequently used in photobiological experiments after a single UVB irradiation. We wanted to standardize the measurement of photosensitivity of mice with different genotypes and to determine the minimum dose required for the onset of skin symptoms in any given mouse strain.

We wanted to clarify what differences one can see among the SKH-1, C57BL/6, and Balb/c mouse strains in terms of skin response to UV radiation and how they could be detected by imaging methods. We wanted to investigate the appearance of erythema, the extent of edema, and the number of inflammatory cells in different mouse strains as part of the skin reaction.

We also aimed to investigate changes by histological, immunohistochemical, and cytokine expression measurements to clarify the mechanism why the mouse skin reacts differently to UVB than humans.

We set out to investigate the use of phototherapy at the Department of Dermatology, Venereology and Dermatooncology of Semmelweis University in patients treated for CD8-positive lymphomatoid papulosis between 2008 and

2015. Our aim was to examine the clinical characteristics, diagnostic difficulties and therapeutic possibilities, with special focus on the effectiveness of phototherapy. We further aimed to obtain data on possible progression, long-term survival and associated malignancies by reviewing patients' medical histories.

We were also interested in the histopathology of this group of LyP cases with rare immunophenotype. Based on our findings, our goal was to classify the significance of CD8-positivity and rethink their classification.

3. Methods

1) Effects of a single UVB radiation on mouse skin

Animals used

In our studies, we used female mice of C57BL/6, Balb/c, and SKH-1 genotypes, 20 for each genotype. Animals were housed under normal animal housing conditions. Their diet was not restricted, they received regular rodent chow. UV irradiation was performed at 8 weeks of age.

UVB-irradiation

In the case of fur-covered mouse strains, the hair was removed 24 hours before the study, and then the skin was irradiated with UVB in six 1x1cm areas. Each area was irradiated with UVB at 40, 50, 60, 80, 100, 150 mJ/cm2. During the treatment, the animals were temporarily anesthetized with intraperitoneal Avertin. For control, a simulated irradiation was performed on the skin of mice treated identically on the same way, but their

skin was covered with a UV-impermeable film that did not adhere to the skin.

Evaluation of erythema and edema

During the experiments, photographs were taken and the intensity of erythema was evaluated by two independent researchers at 24 and 48 hours. To obtain a numeric value, the erythema developed was scored on a scale of 0-3. Photos were also evaluated with Photoshop CS6© for objective measurements. To quantify edema in vivo, skin thickness was measured with a caliper. The thickness of the skin was measured both on irradiated and non-irradiated areas and a quotient was calculated. The most significant changes were seen after 48 hours, so this time point was used as the endpoint in all experiments.

Ex vivo measurement of skin edema with optical coherence tomography

For our OCT studies, we used an RTVue-100 Fourier-domain OCT equipped with a CAM-L corneal lens adapter at 26,000 axial scans per second and an axial resolution of 5 micrometers. Forty-eight hours after irradiation, the animals were anesthetized, skin samples were excised, and OCT measurements were performed on the *ex vivo* samples. Epidermal thickness was determined by blind measurements of the same examiner using the OCT device's built-in software measuring tool. The increase in thickness of different groups of mice were investigated by comparing irradiated and sham-irradiated areas. Mean values were calculated from at least 4 measurements.

Histopathological evaluation

Skin samples measuring 1 x 1 cm were removed from the backs of the mice, each consisted of an irradiated and a non-irradiated part. Samples were fixed with 10% buffered formalin and embedded in paraffin. 4 μm sections were stained with hematoxylin and eosin (H&E). For immunofluorescence measurements, 0.5x1 cm skin samples were taken from separate sites on irradiated and non-irradiated skin surfaces. These were frozen in liquid nitrogen and embedded in Cryomatrix gel. 10 μm thick sections were stained with anti-CD3 antibody. Cell nuclei were visualized with propidium iodide. Furthermore, skin was obtained from all three genotype mice one hour after the UV irradiation at Clinically Relevant Doses, and samples were stained with anti-CPD antibody. The thickness of the epidermis and dermis and the presence of inflammatory cells after H&E staining were examined in the skin, evaluating the cellular composition in five fields of view, both in the irradiated and in the sham- irradiated areas.

Skin homogenization and cytokine assays

Skin samples of 1 cm^2 from the dorsal skin of the animals were taken from two areas: a) that received a dose we called Clinically Relevant Dose (CRD) and b) the highest dose of 150 mJ/cm^2. Skin from mice exposed to simulated irradiation was used as a control. Skin samples were weighed on an analytical scale and dipped in T-PER protein isolation buffer supplemented with protease and phosphatase inhibitors. The skin was homogenized and centrifuged with an IKA Ultraturrax homogenizer, and the supernatant was aliquoted into 55 μl volumes and stored at -80°C until further analysis. Protein concentrations in the supernatants were quantified by microBCA protein assay. Levels of IL-1β, IL-6, IL-10, IL-17A,

IFN-γ, and TNF-α in the lysates were measured by Bio-Plex ProTM mouse Cytokine 6-Plex magnetic bead assay on a Bio-Plex 200 system. 1 mg protein per measurement was used for cytokine analysis. Cytokine assays were performed on two samples each for both irradiated and simulated irradiated skin samples.

Statistical analysis

Statistical analyses were performed using Real Statistics, MedCalc, and OriginPro software. Normality was assessed by Saphiro-Wilks test. Groups were compared by one-way ANOVA and unpaired T-tests. For non-normal distributions, the non-parametric Kruskal-Wallis test and pair-pair comparison were performed using the Mann-Whitney U-test.

2) Clinical and histological examination of CD8 + Lyp patients

Patient samples, diagnosis

After reviewing the period from 2008 to 2015 at the Department of Dermatology, Venereology and Dermatooncology of Semmelweis University, we could identify 14 patients who have been diagnosed with CD8+ lymphomatoid papulosis. Their clinical material was re-evaluated and repeated histopathological examinations were performed. The diagnosis of LyP was based on the clinical features of the disease and on the histopathologic criteria defined by WHO-EORTC and WHO classification. Thirdly, the diagnostic recommendations of the Cerroni manual has also been followed. The patient's examination consisted of a physical examination, routine laboratory tests, a chest X-ray, and a CT scan.

Test samples

Flow cytometry and TCR gamma gene rearrangement studies were performed to assess peripheral blood involvement. Histological evaluation was performed on formalin-fixed, paraffin-embedded samples. We were able to re-examine the histological specimen from each of the 14 patients, which histological reassessment was performed by two haematopathologists.

Histopathology

Upon examining sections of LyP specimens stained with HE, the following histological abnormalities were assessed: ulceration in the epidermis, spongiosis, parakeratosis, vacuolar degeneration of the basal layer, degree of epidermal hyperplasia, and degree of epidermotropism as main parameters. In the latter case, the degree was considered as follows: mild, when the ratio of lymphoid cells/10 keratinocytes was 1-5, moderate: 6-15 and strong epidermotropism at more than 15 lymphoid cells/10 basal keratinocytes. In addition, the shape and nature of the dermal infiltrate (superficial, wedge-shaped, perivascular, periadnexal, granulomatous), vascular involvement (vascular wall damage, and erythrocyte extravasation) were evaluated, along with the involvement of the subcutaneous tissue. We also examined the size of the epidermotropic and lymphoid cells in the dermis, as well as the other cellular composition of the infiltrate.

Processing of immunohistochemical and molecular genetic samples

The following antibodies have been used for immunohistochemistry: CD3, CD20, CD30, Ki67, granzyme B,

MUM1, CD4, CD8, CD5, CD7, CD2, TIA-1 and PD1. Human tonsilla samples were used as controls. In six cases, molecular assays could also be performed by isolating DNA from frozen skin samples or paraffin-embedded biopsy material. The BIOMED-2 Concerted Action protocol was used for TCR γ gene rearrangement PCR assays. Cases reported as CD8+ LyP were collected by reviewing English journals indexed by Medline from 1990 to January 2016.

4. Results

1) Effects of a single UVB exposure on mouse skin

Dark-haired C57BL/6 mice are more sensitive to UVB radiation than albino Balb/c and SKH-1 mice

Significant differences were found in the doses required for a barely perceptible skin change in mice with different genotypes. 48 hours after UVB irradiation, abnormalities were observed in C57BL/6 mice at 41.4 mJ/cm^2, which is significantly lower than the mean CRD value measured in Balb/c (54.3 m J/cm^2). In contrast, SKH-1 nude mice showed skin symptoms at only 92.5 mJ/cm^2.

Erythema at CRD is more pronounced in C57BL/6 and Balb/c mice than in SKH-1 mice

C57BL/6 and Balb/c mice displayed a moderate degree of erythema, in contrast to SKH-1 type mice, where little redness was observed, their skin reaction mainly consisted of edema. Because MED only describes erythema, we created a new term: Clinically Relevant Dose (CRD). This indicates the lowest dose at which a clinically detectable symptom occurs in the form of erythema, edema, or a combination of the two. The strongest

skin reaction has been observed after 48 hours in all mouse strains. Objective analysis of erythema was performed on photographs by Photoshop CS6, and a new parameter, the relative erythema quotient was created to numerically express the degree of redness. The erythema intensity was significantly higher in the C57BL/6 and Balb/c groups than in the SKH-1 mice, but there was no significant difference between the two fur-covered mouse strains. After an irradiation with CRD, there was no significant difference in the number of CPD-positive cells, that confirms that these doses result in a comparable degree of UV-damage at the cellular level, despite the fact that the absolute values of the doses are different.

Skin edema is a marker of similar importance to erythema in the response of mouse skin to UVB radiation

To numerically describe skin thickening, we constructed a measure, the skin edema ratio. The rate of skin edema in C57BL/6 mice was 1.46, that equals to a 46% thickening. In the Balb/c and SKH-1 groups, the corresponding values for CRD were 21% and 72%, respectively. In addition, skin thickness was determined by *ex vivo* optical coherence tomography to find that the response of mouse skin to UV is associated with a significant degree of dermal edema after an irradiation with Clinically Relevant Doses. When the degree of skin thickening was calculated from the OCT measurements, similar values were found as with the caliper measurements. C57BL/6 mice showed a 1.61-fold increase in skin thickness, while Balb/c mice had a 1.29-fold increase after CRD. The most profound change in thickness was observed in SKH-1 mice, with a 1.63-fold value. Analyzing skin reactions at CRD irradiation, the following differences were observed: erythema was stronger in

Balb/c mice (52.85% erythema vs 47.15% edema) and in C57B/6 (44.48% erythema vs 55.52% edema). In SKH-1 mice, edema dominates the skin changes after UVB (41.98% erythema vs 58.02% edema).

Increase in skin thickness at clinically relevant doses is proportional to inflammation

Since edema does not appear during MED testing in human skin, we wanted to further investigate this phenomenon in the mouse model. Samples from the dorsal skin of mice irradiated with CRD were subjected to histological examination and the observed abnormalities were compared to non-irradiated dorsal skin. The degree of inflammation was expressed by determining the number of inflammatory cells after irradiation of each strain with CRD. The results showed that relatively few inflammatory cells were detected in C57BL/6 (40±10 inflammatory cells/mm^2) and Balb/c (38±15.41 cells/mm^2) mice. In comparison, a significantly higher degree of cellular infiltration was observed in the SKH-1 group (332 ± 136.63 cells/mm^2). Most skin infiltrating cells were neutrophilic leukocytes, but lymphocytes were also present in increased numbers. Using the anti-CD3 immunohistochemical reaction, we confirmed that the rate of lymphocyte influx into the dermis was also higher in CRD in hairless mice than in hair-covered animals. Sections were also stained with toluidine blue to detect mastcells, however, the number of these cells was no higher in irradiated skin than in non-irradiated skin. The degree of skin thickening in H&E-stained sections was also assessed by measuring the thickness of the epidermis and the dermis. In this study, a 1.45-fold increase was observed in C57BL/6 mice and a 1.29-fold increase was detected in the Balb/c group. The greatest increase

of 1.75-fold (SD ± 0.20) was observed in the SKH-1 strain. When we studied the epidermis, the most marked hyperplasia was observed in nude mice: it was 2.26-fold, compared with 1.4-fold in C57BL/6 mice and a 1.21-fold change in the Balb/c group.

At 48 hours after UV irradiation, IL-1β and IL-6 levels increase significantly

In order to identify the major signal that attracts inflammatory cells to the skin, levels of various cytokines were determined from UV-irradiated skin samples. The most significant changes after a single irradiation were observed in IL-1β and IL-6 levels. At high doses, IL-1β was the only cytokine that showed a strongly significant increase in all genotypes when compared to controls. The increase in IL-6 levels was significant only in C57BL/6 mice at the maximum dose of 150 mJ/cm^2 after 48 hours. There was no additional significant difference between genotypes, although their CRD values were numerically different. Cytokine levels of TNF-α, INF-γ, IL-10, and IL-17A did not show a positive correlation with UVB. For these cytokines, the basal expression levels of the sham-irradiated groups were relatively high and there was no significant difference between genotypes.

2) Clinical and histological evaluation of CD8+ Lyp patients

Within the CTCL group, the majority of LyP cases have CD4+ immunophenotype, although in the recent years occasional case reports have been published about the far less common CD8+ variants. After a thorough review of cases in our clinic, we were able to identify 14 patients with CD8+ LyP between 2008-2015.

The ratio of male to female was 1:1, all patients were Caucasians. At the time of diagnosis, the age of patients ranged from 5 to 69 years, with a median of 30.5 years. The disease appeared with erythematous, necrotic papules, and in two cases blisters and erosions were also observed. Spontaneous regression was observed in all patients, followed by an asymptomatic period of varying length. Eventually, in most cases, the symptoms reappeared. The development of symptoms followed the characteristic time course: multiple lesions appeared at the same time that were primarily located on the trunk and limbs. Twelve of the patients received treatment: phototherapy and/or topical corticosteroids. It is often seen in the clinical practice that the diagnosis of LyP is challenging, the time between the onset of clinical symptoms and the proper diagnosis was between 1 and 7 months (mean 2.62±2.26 months). All patients were free of associated systemic symptoms. No lymph nodes with abnormal morphology were noted, neither were the peripheral blood or internal organs involved. Two patients had an associated lymphoproliferative disease in addition to LyP. One patient was previously diagnosed with CLL, with associated lymph node involvement that was histologically verified. Another patient had previously been confirmed to have MF, stage 1B, with a CD8 positive/CD30 negative immunophenotype. 8 years after the diagnosis of MF, he developed LyP with papules localized to the thigh. Two patients were diagnosed with prostate cancer during the follow-up of LyP. When we looked back at the treatment of LyP, we saw that the symptoms mostly regressed with skin-directed treatment. Patients received topical steroids and various forms of phototherapy. In 2 cases, the LyP resolved without treatment, although home sunbathing was

recommended for these patients. During the follow-up these individuals did not require further treatment. At the time of publication, all patients were alive, with a follow-up time of 5 to 74 months (mean 25.69 months).

Histopathological evaluation

We found one case of type A Lyp characterized by mild epidermotropism of small lymphoid cells and large number of scattered atypical large cells in the dermis in a reactive background consisting of mainly small lymphoid cells and histiocytes resembling to type A LyP. Eosinophils and neutrophils were virtually absent. In 4 cases of the 14 total, LyP B was confirmed: the histological appearance of the samples were indistinguishable from CD8 positive MF with small, slightly atypical tumour cells, moderate epidermotropism and superficial, band-like or perivascular dermal infiltrate, without epidermal changes in 3 of these patients. Two of our 3 juvenile cases belong to this MF-like histologic type. One another case with sheets of anaplastic large cells in the dermis, mixed with eosinophils and minimal epidermotropism were best classified as LyP type C. Seven cases from our collection were characterized by typical microscopic features of LyP type D with a combination of epidermal hyperplasia and prominent epidermotropism of atypical small to intermediate sized cells with mild nuclear atypia associated with parakeratosis, spongiosis, vacuolar degeneration of the basal keratinocytes and ulceration. The dermal infiltrate was wedge shaped with perivascular accentuation, extending into the subcutaneous fat in 2/7 cases, made up mostly small reactive lymphoid cells with scattered atypical mid-sized cells and few aggregates of large cells in 2/7 cases. The number of histiocytes was low and lack

of plasma cells was a constant feature, eosinophils were found only in one case. Angiocentricity in conjunction with red cell extravasation and perieccrine infiltration were found in 3/7 cases. One case exhibited wedge shaped dermal, significant perifollicular infiltrate and strong folliculotropism without follicular mucinosis and strong epidermotropism consistent with type F LyP.

Immunohistochemical findings

The atypical lymphoid cell component was positive for CD8 in all cases. CD30 positivity of 10 to 70% of CD8+ atypical cells was detected with membranous staining pattern and Golgi accentuation in 12/14 cases. CD30 positive cells were localized both in the dermis and in the epidermis in 9/14, exclusively in the dermis in one case, and in the epidermis in two cases. Two type B cases were CD30 negative even after careful repeated examination. The CD30 expression of the small/medium sized epidermal infiltrate in types D and B was weaker in all positive cases compared to the CD30 expression of the dermal large cells in types A and C. In two cases, the intra-epidermal atypical cells co- expressed CD4 weakly in 10-20% of the cells. CD7 antigen loss was seen only in one case. CD2 and CD5 expressions were preserved in all the tested cases. We observed at least one cytotoxic marker positivity (TIA-1 and granzyme-B) in all but one case. PD1 expression was detected in 3/12 of our cases and the expression was not associated with histological type: 1/4 type B and 2/5 type D cases were positive. At least focal MUM1 expression was found in 9 out of 12 of our cases, however, >20% positivity was detected in 2 out of 12 cases and none of the type D cases demonstrated significant MUM1 expression, only 15-20% of the cells were positive in two cases.

Molecular findings

Testing for T-cell receptor gamma gene rearrangement was performed in 6 cases. Monoclonal T-cell gene rearrangement was detected in 3 cases while polyclonal features were identified in the remaining 3 cases. In one case with previously diagnosed MF, clonal T cell population was not detected in any of the specimens.

Treatment of CD8+ LyP with phototherapy

Phototherapy was used in 7 cases in the treatment of the 14 patients. The follow-up time was significantly longer in patients who received phototherapy. Their mean follow-up was 32.7 months, compared with a mean of 17.5 months for patients who did not receive phototherapy. Follow-up data showed that 6 of the 7 patients treated with phototherapy achieved a complete response (CR) and 1 patient a partial response (PR).

5. Conclusions

1) Single UVB radiation on mouse skin experiments

We were the first to study the skin response of mice from different genetic background to a single UVB irradiation under standard conditions, in vivo and in vitro. We found that different mouse genotypes exhibit different clinical symptoms after the same dose of UVB irradiation.

We observed that black-haired C57BL/6 mice were more sensitive to UVB irradiation than albino Balb/c and hairless SKH-1 mice. In their case, the dose of UVB required to develop a barely perceptible skin symptom was 41.4 mJ/cm^2, compared to a 31% higher dose for Balb/c mice and 120% higher dose for SKH-1 mice.

We demonstrated that the term “MED” is inadequate to describe real clinical observations when studying mouse skin. This is the case, because the UVB response of mouse skin depends greatly on the genotype but it always consists of some degree of edema. We have developed a new concept, the clinically relevant dose (CRD), which is defined as the lowest dose at which erythema or edema is first seen after a single UVB irradiation. We suggested that this terminology be used instead of MED in experimental setting on mice.

After irradiation with the Clinically Relevant Dose, the skin thickness increased, and so did the number of inflammatory cells that infiltrates the skin. Most of these cells were neutrophilic leukocytes and lymphocytes in our studies. The hairless SKH-1 group had the highest degree of cellular infiltration.

When we examined the cytokine level changes of UVB-irradiated skin samples, we concluded that IL-1β increases in all genotypes compared to the control after both CRD and maximal irradiation at 150 mJ/cm^2. Another cytokine, IL-6, was also found to be elevated, however, only in C57/BL6 mice and only after the highest dose of UVB irradiation. This observation supports the universal role of IL-1β and the genotype-specific role of IL-6 in acute UVB-induced inflammation.

Although there was a significant difference in the UV tolerance of each strain, i.e., the dose of UV radiation required for minimally detectable skin lesions, no significant difference in cytokine expression levels was observed between the strains tested. Consequently, the clinical differences observed in CRD values can’t be explained by the differences observed in cytokine expression.

We found that changes of cytokine levels in mice were similar to those observed in human UVB experiments.
We found that the use of the mouse strain C57BL/6 seems to be the most appropriate for modeling the physiological responses of human skin. C57BL/6 respond to UVB predominantly with erythema, similarly to what we see in the human setting.

2) Clinical and histological findings in CD8+ Lyp patients

In our patient collection we found 14 patients who were diagnosed with a rare, CD8-positive variant of LyP. Although CD8+ LyP cases are traditionally classified as type D, not all of our cases belonged to this type based on the observed histological features.
By reviewing the clinical data of the patients and monitoring them for a long time, we concluded that phototherapy is an effective treatment for CD8+ LyP types.
We observed that the onset and dynamics of clinical symptoms of both CD4+ and CD8+ Lyp were similar. No different clinical presentation or different response to therapy was observed for each histological type of CD8+ Lyp.
We also reviewed the histological features of the patients, classified them by type, and proposed clarification of the LyP classification. In our view, linking type D Lyp and CD8 positivity at the definition level is inadequate because several other subtypes also have CD8+ variants. It is recommended that type D be used for CD8+ Lyp with the morphology of primary cutaneous aggressive epidermotropic T-cell lymphoma in order to make the histopathological distinction, and thus the diagnosis, more straightforward.

We found that although histopathological findings are important in the final diagnosis of CD8+ skin disease, it is the clinical appearance and the course of the disease that determine the correct diagnosis of the condition. We emphasized the importance of follow-up among these patients in order to diagnose any associated malignancy as soon as possible. Associated oncologic diseases were observed in four of our patients, two of which were rare findings, prostate cancer, according to the literature.
To the best of our knowledge, we were the first to report a case of a CD8 positive variant of type F lymphomatoid papulosis.

6. List of publications

Publications related to the thesis:

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