A-4 Studies on Health Effects by the Increase of Ultraviolet Ray
A-4. 6. Studies on the effects of oxidative stress due to UV-B irradiation

Contact person: Margarita Dimitrova Apostolova, Ph.D
Noriko Nishimura, Ph.D
Chiharu Tohyama, Ph.D
National Institute for Environmental Studies
Environmental Agency
Onogawa 16-2, Tsukuba, Ibaraki, 305 Japan
Tel:+81-298-50-2336, Fax:+81-298-56-4678
e-mail: ctohyama@nies.go.jp

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Abstract

Ultraviolet B radiation results in the generation of oxidative stress in the tissue. Understanding the mechanisms of antioxidant self-defense system and their role in skin maintenance and function as well as the role of metal-dependent systems in responding to ultraviolet radiation may offer a new approach for preventing and/or treating radiation injury. In order to elucidate how antioxidant system is involved in this process, we have studied possible protective roles of metallothionein in the skin against daily UV-B radiation and the eventual compensatory mechanisms by utilizing transgenic mice whose MT gene were knocked out by homologous recombination. It was found that MT-null mice were less sensitive to UV-B radiation in comparison with their ancestors C57BL/6J and 129-SV mice. The MT-null mice contained the lowest skin NPSH levels compared to the other two mice strains, but had the greatest GSH-Px activity after ultraviolet radiation. A dose-dependent increase of TBA-RS were found in MT- null mice in comparison with C57BL/6J and 129-SV mice. Taken together, the present results indicated that the sensitivity of the skin to UV-B irradiation was not necessarily associated with the endogenous MT synthesis, but that the protective role of MT against free radical could not be excluded.

Key Words Metallothionein, UV-B, MT- null mice, interleukins

Introduction

Depletion of atmospheric ozone may result in an increased exposure of human to ultraviolet (UV) radiation[1),2)]. A proportional increase in UV radiation on the ground level was measured during the last four winters. Harmful effects of UV radiation have been stuidied for risk estimation of higher exposure of man to UV radiation with respect to skin cancer. Ultraviolet radiation is the primary cause for non-melanoma skin cancer. Exposure to UV induces sunburn, and erythema promotes premature aging of the skin and causes ocular damage. Especially UV-B (280-315 nm) is considered to be responsible for the harmful effects [3)-6)]. Much of our understanding of the mechanisms of skin phototoxicity has evolved from studies of the effects of ultraviolet radiation on various target molecules critical to normal cell function. Among them, widely studied targets are nucleic acids, since damage to the genome has obvious implications in explaining the mutagenic and carcinogenic properties of UVR. Various enzymes and proteins which may be induced or inactivated by UVR, and lipids, which can undergo radical attack leading to the lipid peroxidation, have to be studied._There is considerable evidence that exogenous antioxidants, radical quenchers, and thiols may have a role in cancer prevention[7)]. It is likely that glutathione fulfills similar roles in the skin, acting as an endogenous antioxidant and photoprotective agents. Substantial amounts of glutathione can be formed by recycling of oxidized glutathione (GSSG), catalyzed by the NADPH dependent flavoprotein glutathione reductase[8)]. This reaction is of grate importance since GSSG is produced in many of the chemical and enzymatic reaction of glutathione. In its protective roles glutathione may act as a radical scavenger *per se*, or as a cofactor for protective enzymes. Such

enzymes include: glutathione peroxidase, transhydrogenases, by which, for example cystenyl disulfides are reduced, and glutathione transferases important in drug detoxification processes. These glutathione dependent enzymes and pathways are reviewed in Meister [9] and Meister and Anderson [8].

Other lines of evidence support the fact that in the skin the immune system is a target for UV-B radiation[10]. Zinc is involved in various aspects of immune system expression, in regulation of hormone activity (thymopoetin) and T cell function[11]. The skin thus seems to be sensitive to the Zn status . It has been already shown that topical Zn application induces MT in the skin [12], and both of them have a similar distribution in the basal cells of epidermis. Other correlation was found with MT localization in the skin and the germinative role of the cells in inflammatory skin disease [13] and in the hear follicles of normal skin [14].

Metallothionein is a small molecular weight protein with a very high capacity for binding of metal ions, Zn^{2+}, Cu^{+} and Cd^{2+} [15,16]. There is much evidence that MT plays role in Zn homeostasis in biological systems and that MT biosynthesis can be induced by these metals[17]. The syntheses of MT has been shown to increased by the inflammatory cytokines (IL1b, IL6 and TNFa)[18] and by free hydroxyl radicals [19]. It is relevant that these cytokins are released from damaged keratinocytes and are also involved in the suppression of T cells mediated immunity caused by UV-B. Extracellular MT has been shown to be potent inducer of lymphocyte proliferation[20] and an activation of MT gene is one component of the UV-B response[18]. In this respect it is interesting to study the relationship between MT, UV-B and Zn in the mice with null mutation for MT 1 and MT 2 loci[21].

Research Objective

The aim of this study was to investigate how UV affects antioxidant self-defense system, focusing on metallothionein, glutathione and relevant enzymes, which are involved in scavenging free radicals, and elucidate possible participation of MT by utilizing MT-null mice.

Research Method

Chemicals. The b-NADPH, glutathione reductase and xanthine oxidase were received from Oriental Yeast Co. IL1b and TNFa assay systems were received from Amersham Life Science. Xantine and Cytochrome C were obtained from Sigma. All other reagents were obtained from Wako Pure Chemical Ind., Osaka, Japan. The aqueous solutions were prepared using purified water.

Animals. Female C57BL/6J and 129-SV mice were obtained from Japan Clea Company. Female mice with a null mutation at the MT 1 and MT 2 loci were produced by Michalska and Choo[21]. Mice weighting 18-20 g (8 weeks old) were housed of 10 per cage in an environmentally controlled room (light 7-19 h, 23±1.5 °C, humidity 55±5%). Animals were allowed access to water and a commercial laboratory chow (Japan Clea Company) *ad libitum*. The mice were randomly divided into 4 groups: Control (n=10), 1 day radiation (n=10), 3 days radiation (n=10) and 7 days radiation (n=10). All procedures with these mice were in accordance to Helsinki accord on the use of animals in research.

UV-B Irradiation. The mice were irradiated with UV lamps (FL15E, 280-400 nm, max 312 nm; NIS Com., Japan) in cages (20x15x5 cm) containing 5 animals each. The animals were exposed to UV-B at a daily radiation dose of 2 kJ/m^2, for different time - 1 day, 3 days and 7 days. The exposure time was less than 10 min and no detectable heat was observed. UV-B dose was determined at 312 nm with an RMX radiometer (ATTO, Tokyo).

Collection of tissue and serum. Mice were killed under anesthesia at the 24 hour after the last irradiation. At the time of killing, blood was collected by cardiac puncture, centrifuged at 3,000 **x** ***g*** for 10 min to separate serum. Part of irradiated skin was quickly removed and stored at -80 °C until analysis.

Determination of MT and Zn. The skin was homogenized in 10 % (w/v) of 0.1 mol/L Tris/HCl buffer, pH 7.4 with Polytron homogenizer. The homogenate was centrifuged at 10,000 **x** ***g*** for 10 min and a part from the resulting supernatant was heated for 1 min in a boiling bath, followed by an immediate cooling in an ice-bath. The precipitate was removed by centrifugation at 40,000 **x** ***g*** for 20 min, and the supernatant was used for MT determination by radioimmunoassay (RIA) as described by Tohyama and Shaikh[22].

In order to analyze Zn, skin weighting about 0.1 g was digested with acid mixture (HNO_3:$HClO_4$=3:1). After digestion, inorganic residues were dissolved in ultrapure water, and metal analysis was carried out by inductively-coupled emission spectrometry.

Histochemical analysis. The dorsal skin was fixed in HistoChoice. Deparaffinized 5 mm thick tissue sections were subjected to immunohistochemical staining with ABC method[23]. Antisera against rat MT were produced in rabbits, according to the same method as described earlier [22]. In brief, tissue sections were immersed in a 0.5 % periodic acid solution for 10 min to mask endogenous peroxidase activity, rinsed with PBS, and treated with 5 % control goat serum for 60 min. The sections were overlaid with anti- MT antiserum at room temperature for 1 h and rinsed with PBS. The sections were treated with biotinylated goat-anti-rabbit IgG for 1 h, washed with PBS, treated with the ABC complex for 30 min. The color was developed with TrueBlue Peroxidase substrate. The samples were washed with running tap water, dehydrated and mounted. Control sections were treated with non-immune rabbit serum instead of the rabbit serum.

Analyses of superoxide dismutase and thiobarbituric acid- reactive substances. The activity of superoxide dismutase (SOD) was analyzed by the method of McCord and Fridovich[24] with some modifications. The SOD samples activity was calculated as units according to 50% inhibition. The plasma thiobarbituric acid-reactive substances (TBA-RS) concentrations were measured using a commercial kit.

Enzyme activities and non-protein sulphydryl analyses. The glutathione peroxides activity (GSH-Px) was measured by the method of Lawrence and Burk[25]. Catalase activities were measured by the method of Cohen at all.[26] and non-protein sulphydryl groups were detected by the method of Riddles at all[27].

Statistics. Data were analyzed by an one-way ANOVA, followed by Bonferroni two-tailed t -test. The 0.05 level of probability was used as the criterion of significance.

Results

Metalothionein level in the skin and its immunohistochemical localization. The UV-B irradiation induced MT in the skin of C57BL/6J and 129-SV mice with a maximum concentration on the 3th day, but did not induce MT in the skin of MT-null mice. The control MT level in the skin of C57BL/6J and 129-SV mice was about 15 ng/g tissue compared with that in MT- null mice (<20 ng/g tissue).

In the skin from non-irradiated C57BL/6J mice, immunostaining of MT (isoforms 1 and 2) was observed in hair follicle cells (Figure 1; C57BL/6J, C) in contrast to MT- null mice where this staining was not found. On Day 1 of UV-B irradiation we did not observe a significant difference from the 0-time control animals. On Day 3, the UV-B irradiation caused thickening of the epidermis and dermis, and the structure of basal cell layer was dearranged in all groups of mice studied. The cells in the epidermal basal cell layer, peripheral follicular and sebaceous epithelia, and hair matrix cells had strong MT staining in C57BL/6J mice (Figure 1, 3 d). Both the cytoplasm and the nucleus were stained. In the dermis, macrophages and fibroblasts that had strong MT staining were present. The MT staining was observed only in C57BL/6J mice. On Day 7, some parts of the epidermis from C57BL/6J mice (Figure 1, 7 d) showed thickening which was not as conspicuous as found in UV-B irradiated mice on Day 3 in contrast to MT-null mice where slight thickening was observed sporadically in the skin tissue. Strong MT staining was found in basal cells due to MT overexpression and MT- positive macrophages were observed in the dermis in C57BL/6J.

Level of lipid peroxidation and SOD activity in the skin. The basal level of lipid peroxidation, as indicated by TBA-RS, was greater in the MT- null mice than that in C57BL/6J and 129-SV mice. One day irradiation increased the skin TBA-RS level in C57BL/6J mice more than MT- null and 129-SV mice. The increase of TBA-RS in MT-null mice was further elevated in a dose-dependent manner on Day 3 and 7 of the irradiation in comparison to C57BL/6J mice , where the TBA-RS level was recovered to the basal level on Day 7. No significant changes in TBA-RS concentration were observed in 129-SV mice after different time of treatment.

The SOD activity in the skin decreased significantly in all groups of mice after UV-B irradiation for 1 and 3 days in comparison to untreated control mice (Day 0). There was not a significant decrease of SOD activity on Day 7 of treatment. We did not observed statistical

significant differences in SOD activity, between all strains of mice, on Day 0, 1 and 7 of irradiation.

Non-protein sulphydryl concentrations. The basal NPSH concentration was 6.7±0.7 mmol/ g tissue for MT- null mice in contrast to 13.4±0.6 mmol/g tissue and 10.8±0.2 mmol/g tissue in C57BL/6J and 129-SV respectively. UV-B treatment significantly lowered skin NPSH concentrations in all strains of mice. The decrease in skin NPSH group by UV-B irradiation was greater in 129-SV and C57BL/6J mice than in MT- null mice until Day 7. The UV-B dose decreased the NPSH concentration in 129-SV and C57BL/6J mice in a time-dependent manner in contrast to MT-null mice on Day 1 and 3. On Day 7 of irradiation, the NPSH level was not changed.

Glutathione peroxidase and Catalase activity in the skin . Time course of glutathione peroxidase (GSH-Px) activity in mouse skin after irradiation with UV-B. Two kJ/m^2 led to a time- dependent significant increase of GSH-Px activity in the skin from MT- null mice with a maximum value of 4.7±0.1 IU/ g tissue, achieved on Day 3 of treatment ($p<0.05$). In the experiments with UV-B irradiation of C57BL/6J and 129-SV mice only a slight increase in the GSH-Px activity was observed on Day 1. Thereafter, the GSH-Px activity decreased and reached 0-time control value by Day 7 for C57BL/6J mice. We did not observe a significant difference in GSH-PX activity between 0-time control animals of all groups.

The catalase activity was between 0.05-0.175 activity/g tissue in untreated animals. After different irradiation times, the catalase activity was not significant ly elevated.

Serum levels of IL1b and TNFa following UV exposure.

UV-B irradiationon resulted in a dose-dependent increase of serum TNFa concentration found in all groups treated under this condition. The major difference between strains was apparent from these data, namely that MT- null mice required less UV-B than C57BL/6J and 129-SV mice for the increasing of TNFa concentration. On Day 7 post-irradiation, all mice showed a maximal TNFa level. We found significant difference between TNFa concentration in 0-time control animals from MT- null group and MT positive group.

The untreated C57BL/6J mice showed the lowest serum IL1b level (11±0.8 pg/ml). The IL1b concentration in remaining sera ranged from 10.0±0.4 pd/ml to 8.6±0.2 pg/ml for C57BL/6J mice after different time of treatment. In contrast to C57BL/6J mice, wherever dose-dependent decrease was observed, MT- null and 129-SV mice recovered the IL1b level on Day 3 of the irradiation.

Discussion

UV-B radiation damage is known to be caused by intracellular free radical formation such as superoxide anion and hydroxyl radicals. The UV-B radiation with or without exogenous photosensitezers can cause fluctuations in epidermal and dermal NPSH levels. There are several mechanisms involved in the depletion. More rapid depletion of NPSH after UV-B irradiation was found in C57BL/6J and 129-SV mice in comparison with MT-null mice This decrease may reflect leakage or export of NPSH or GSSG from the cells, transient sequestration of NPSH, e.g. by the formation of mixed disulfides or transient fluctuations in NADPH availability. These processes may occur faster in MT positive mice than that MT-null mice. Induction of "acute" phototoxic responses in the skin such as edema and erythema and changes in the SOD and GSH-Px activity were observed. The GSH-Px level was altered more markedly in MT-null mice than C57BL/6J and 129-SV mice. Our results demonstrate that the enhancement of GSH-Px activity causes resistance to the cytotoxic effects of hydroperoxides in MT-null mice only. Interestingly we did not find a difference in the degree of protection from hydrogenperoxide damage in both MT positive animals , C57BL/6J and 129-SV mice. The results of GSH-Px level taken together with the activity of other enzymes studied here suggest that a relatively small amount of GSH-Px is necessary for maximal protection against exogenous hydroperoxides produced by UV-B irradiation.

We have observed a significant suppressive effect of UV-B irradiation on SOD activity in all groups of mice studied. However, CuZnSOD is co-regulated with MT at the transcription level in yeast, suggesting that metal buffering role of the enzyme can be a part of its antioxidant activity[28]. Under physiological conditions, half-life of the superoxide radicals is about 5 seconds[29]. Hence, in compartments with low SOD activity, superoxide radicals can be also dismutated nonenzymatically to hydrogen peroxide. The hydrogen peroxide is significantly stable and it is further metabolized by GSH-Px and catalase. This experiment showed low

catalase activity for MT-null mice and C57BL/6J mice in comparison with 129-SV mice on Day 0 and 1 post irradiation. The catalase activity together with GSH-Px activity appears to compensate each other in scavenging of hydrogen peroxide in the skin compartment of UV-B irradiated mice.

We observed that NPSH depletion occurring at lower irradiation doses may reflect the protective activities of NPSH groups, and may reflect the overload and cytotoxicity at higher doses. NPSH decrease which we observed only in MT positive group could effectively enhance or prolong phototoxic reactions at many levels, since it is involved directly or indirectly in many pathways pertinent to phototoxicity. The glutathione concentration is within the range causing inhibition of melanin synthesis[30] and its acute or chronic depletion may facilitate enhanced melanin synthesis. Since GSH is a major free thiol, its depletion may expose thiol-containing proteins to insult from UV radiation; Repeated UV exposure could result in accumulating damage to, or aging of, structural protein. Thus the observed depletion of NPSH groups could potentiate long-term biological effects of UV-B radiation such as increased pigmentation, enhanced aging of the skin, and skin cancer induction. Turrell and Pidoux[31] found that human skin cell lines become strongly sensitive to UV-B radiation following extensive GSH depletion. They observed a quantitative correlation between cellular GSH content and skin sensitivity to UV-B. In the present experiment, C57BL/6J and 129-SV mice exhibited the highest endogenous NPSH content but were more sensitive to UV-B irradiation in contrast to MT-null mice with a low endogenous NPSH content. This results suggest that the resistance of MT-null mice to UV-B irradiation does not correlate with the endogenous NPSH content.

The UV-B radiation exerts lipid peroxidation and subsequent tissue damage by oxidative stress. MT may be induced in response to oxidative stress and may protect tissues from oxidative damage. In fact, the skin MT concentration induced by oxygen radical generation was well correlated with lipid peroxidation levels, as shown by measurement of TBA-RS. UV-B treated MT-null mice showed high level of TBA-RS and absence of MT in the skin in comparison with C57BL/6J and 129-SV mice, suggesting that the increase of lipid peroxidation might be related to skin MT induction. To clarify the relationship between lipid peroxidation and MT synthesis immunohistochemical staining of skin sections was done. The UV-B irradiation of C57BL/6J mice increased MT level in the basal cell layer, peripheral follicular and sebaceous epithelia. In the corium of this mice macrophages and fibroblast cells with strong MT staining was found in contrast to the results observed in MT- null mice. The epidermis of three days irradiated C57BL/6J mice has 2 to 3 layers more than epidermis from MT-null mice, indicating that the thickening appearing as a result of UV-B irradiation was greater in MT positive mice. On Day 7, some parts of the epidermis contained increased layers and showed thickening which was not as conspicuous as found in Day 3 of irradiation. These observations suggest that MT is directly or indirectly associated with development of epidermal keratinocytes. The present study showed that MT was located in the cytoplasm and/or nucleus depending on cell types and conditions. In this experiment MT was found in macrophages which appeared during UV-B radiation in the corium of C57BL/6J mice in contrast to undetectable macrophage MT level in MT-null mice (Figure 1). The appearance of macrophages in the corium and increase of TBA-RS levels in the skin samples are essential markers for developing of UV-B toxicity. Inflammatory cells such as macrophages and neutrophils produce hydrogen peroxide and hypochlorous acid and these oxidants may damage bystander cells and may be responsible for much of the damage associated with the inflammatory process[32]. Cytokines such IL1b and TNFa, which are secreted from the macrophages during an immune inflammatory response, are the major mediators of skin acute phase protein synthesis and MT induction. These cytokines are involved in immune reactions after UV-B irradiation[10]. It is known that UV-B irradiation is able to promote the synthesis and secretion of TNFa and IL1b[33]. Our resent study has shown that daily UV irradiation increased TNFa concentration in all strains of mice and decreased IL1b concentration in dose-dependent manner in C57BL/6J mice only. It was also pointed out that studies of MT gene regulation identified the involvement of both cytokines and other immunomodulators which can control MT synthesis[34]. These observation in addition to our results supports the hypothesis that the possible relationship between cytokines, UV-B damaged and MT can not be excluded. The genetical difference between UV-B resistant and UV-B susceptible strains can be found and future study are required to establish the precise mechanisms of UV-B protection in MT-null mice.

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