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Published in:
Experimental Dermatology

DOI:
10.1034/j.1600-0625.2003.120307.x

2003

Experimental Dermatology 2003: 12: 278–282
Blackwell Munksgaard . Printed in Denmark

EXPERIMENTAL DERMATOLOGY
ISSN 0906-6705

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Sonesson B, Rosengren E, Hansson A-S, Hansson C. UVB-induced inflammation gives increased D-dopachrome tautomerase activity in blister fluid which correlates with macrophage migration inhibitory factor.
Exp Dermatol 2003: 12: 278–282. © Blackwell Munksgaard, 2003

Abstract: UVB light was used to induce an experimental inflammation in normal human skin in order to investigate its correlation with the activity of the newly described enzyme D-dopachrome tautomerase (DDT) in the fluid of experimental blisters. Macrophage migration inhibitory factor (MIF) activity was determined as a closely related marker of inflammation. DDT and MIF activities were demonstrated in blister fluids in all 10 healthy subjects. All but one of these subjects showed increased activity of DDT and MIF after three minimal erythemal doses (MED) of UVB. The mean activity of DDT increased approximately twofold and the mean activity of MIF also increased twofold after UVB in our experimental model. We found a strong correlation between DDT and MIF activities. The presence of DDT in epidermis and its increase at UV irradiation was confirmed by immunohistochemical studies. In this study, DDT is for the first time demonstrated in the skin. It is also the first time DDT can be related to inflammation, and its covariation with MIF strengthens this observation.

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Key words: skin –extracellular –cytokine

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Accepted for publication 28 January 2002

Introduction

We recently identified two enzymes that convert the non-naturally occurring D-isomer of dopachrome by a tautomerase reaction to indolic compounds (1). One of these enzymes was found to be identical to macrophage migration inhibitory factor (MIF) (2–4). The other enzyme was a not previously described protein, which was named D-dopachrome tautomerase (DDT) (5). DDT converts D-dopachrome by tautomerization and decarboxylation to 5,6-dihydroxyindole (DHI), and MIF converts D-dopachrome to 5,6-dihydroxyindole-2-carboxylic acid (DHICA) (1) (Fig. 1). The enzymatic activity of MIF as well as of DDT was a new finding, and a search for endogenous substrates showed *p*-hydroxyphenylpyruvate and phenylpyruvate to be substrates of the two enzymes (6, 7).

DDT activity was first observed in cultured melanoma cells (5). Higher levels of activity were seen in human liver and in human blood (5, 8). MIF was first described as a soluble material released from sensitized lymphocytes when stimulated by antigen, but has later on been found to be expressed in a large number of tissues. MIF has been found to potentiate septic shock and to be a glucocorticoid-induced modulator of cytokine production, counteracting the immunosuppressive effects of glucocorticoids (9, 10).

In the early phase UVB exposure of the skin gives an inflammatory reaction, which has been shown to increase the production of several cytokines by the keratinocytes and the infiltration of inflammatory cells into the skin (11–13). *In vitro*

Abbreviations: DDT: D-dopachrome tautomerase; MIF: macrophage migration inhibitory factor; DHI: 5,6-dihydroxyindole; DHICA: 5,6 dihydroxyindole-2-carboxylic acid.

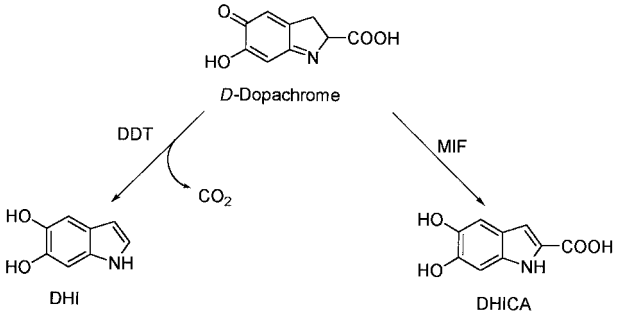

Figure 1. Scheme for the conversion of D-dopachrome catalysed by D-dopachrome tautomerase (DDT) and MIF, giving 5,6 dihydroxyindole (DHI) and 5,6-dihydroxyindole-2-carboxylic acid (DHICA), respectively.

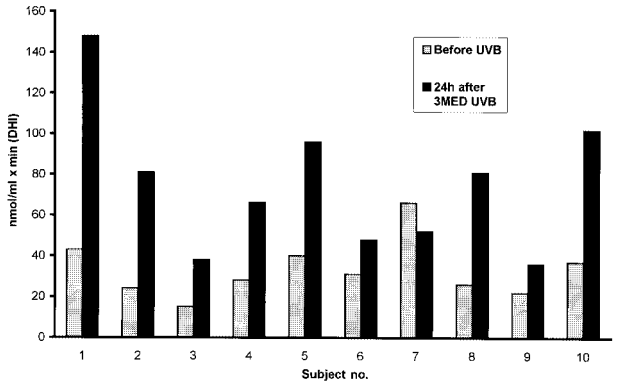

Figure 2. Increased D-dopachrome tautomerase (DDT) activity in human blister fluid after UVB irradiation. Normal skin was exposed to three MED of UVB and experimental blisters were raised 24 h later. DDT activity was determined in the blister fluid. Non-irradiated skin was used as control. The *P*-value in the comparison using the Wilcoxon sign-range test was $P = 0.006$.

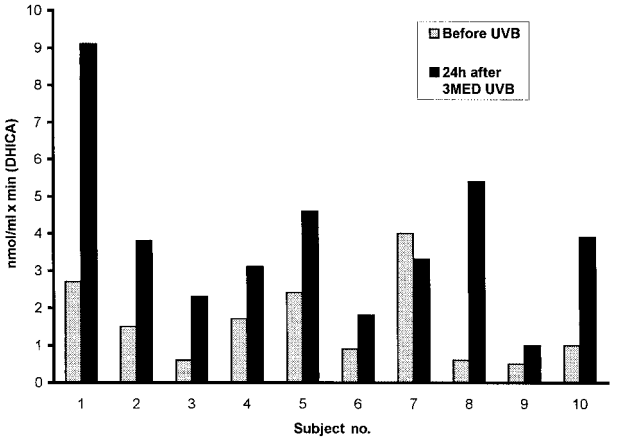

Figure 3. Increased MIF activity in human blister fluid after UVB irradiation. Normal skin was exposed to three MED of UVB and experimental blisters were raised 24 h later. MIF activity was determined in the blister fluid. Non-irradiated skin was used as control. The *P*-value in the comparison using the Wilcoxon sign-range test was $P = 0.006$.

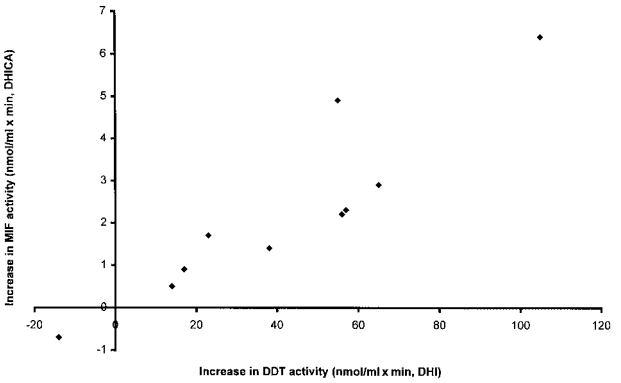

Figure 4. The relationship between the increase in DDT activity and MIF activity after UVB irradiation. Experimental blisters were raised on normal skin with and without exposure to three MED of UVB light. The activities of DDT and MIF were determined in blister fluids and the increase after UVB exposure was calculated.

experiments have shown that keratinocyte-derived cytokines may be available for interactions also below the basement membrane where they can contribute to the elevation of dermal circulation after UVB exposure (14) and play a crucial role in the mechanisms resulting in UVB-induced immunosuppression (15) and tolerance (16). A recent study has shown the enhancement of MIF production by human keratinocytes after UVB irradiation (17).

The suction blister method, a widely used *in vivo* model for studying the influence of external physical and chemical stimuli on the skin, was first described by Kiistala (18). Electron microscope (EM) studies showed that the level of blister separation was between the cell membranes of basal cells and the epidermal basement membrane. Studies on the mechanisms regulating the diffusion of molecules into the suction blister fluid showed a free passage of molecules under 30 kDa and a progressively inhibited diffusion over that size (19). Blister fluid concentrations of total protein, albumin and gamma globulin were all about 25% of those in serum. Some acid hydrolases showed increased values after UVB exposure, but no significant increase in protein concentration in general was shown in suction blister fluid after UVB exposure. The values of sodium, potassium and chloride in suction blister fluids were the same as for serum, also in fluid from blisters raised on erythematous skin provoked by UVB (20, 21). In studies of skin reactions, the analysis of blister fluid has been shown to give more accurate values of locally pro-

duced inflammatory markers than serum analysis (22).

In the present study we used UVB light to induce an experimental inflammation in order to investigate its correlation with the activity of the new enzyme DDT in the extracellular liquid. MIF was determined as a closely related marker of inflammation

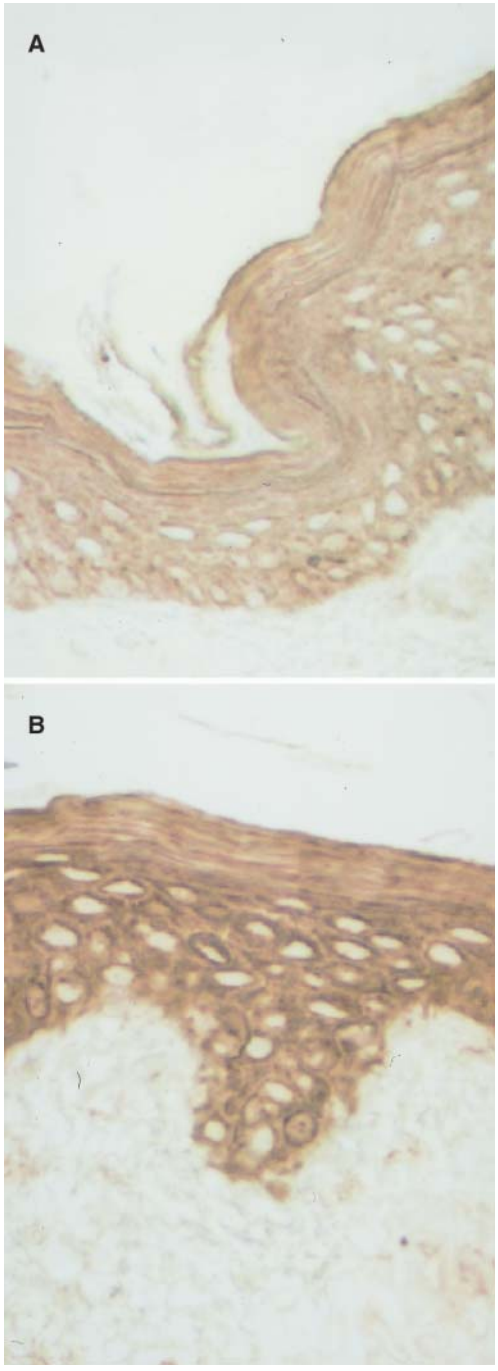

Figure 5. Binding of anti-DDT polyclonal antibodies in epidermis. Immunohistochemical staining in normal skin *(a)* and in normal skin 24 h after three MED of UVB *(b)*.

Methods

UVB radiation

The source of UVB was a unit of 10 fluorescent lamps (Waldmann, UV 3001, F85/100 W-UV6) with an emission spectrum between 285 and 350 nm and a peak emission of 312 nm. The estimated intensity was 1.2 mW/cm^2 at a target distance of 20 cm. The individual minimal erythemal dose (MED) for UVB was determined on the ventral side of the left forearm for each of the subjects. None of the subjects had used a commercial sunbed or acquired a suntan during the previous 2 months. The study was approved by the Ethics Committee of the Medical Faculty, University of Lund, Sweden.

Assay for DDT and MIF

In the dopachrome tautomerase experiments there were two spots (each 5 cm^2) irradiated with three minimal erythemal doses of UVB 24 h before suction blisters were raised on the ventral side of the right forearm of 10 healthy subjects (six men, four women: median age 45: age range 29–68 years). Two non-irradiated sites were included as controls. Blisters were produced according to Kiistala (18) but using disposable devices to avoid virus infections. The blisters were punctured and the fluid was collected, centrifuged ($1000 \times g$) for 1 min and the fluid was analysed immediately after centrifugation. Dopachrome tautomerase activity was determined by measuring the formation of DHI (DDT activity) and DHICA (MIF activity) from D-dopachrome. D-dopachrome was formed from D-dopa by oxidation using sodium periodate (23). Blister fluid to be analysed was immediately added to the dopachrome solution and the formation of DHI and DHICA was determined using high-performance liquid chromatography (HPLC) and a fluorescence detector as previously described (24, 25).

Immunohistochemical analysis

Normal skin on the ventral side of the right forearm was irradiated (5 cm^2) with three MED of UVB and biopsies were taken after 24 h. Non-irradiated skin from the left forearm was used as reference. Tissue samples were snap frozen in isopentane on dry-ice, stored at -70 C and prepared according to standard procedures. Briefly, sections measuring 5 µm were fixed in acetone for 10 min at room temperature and incubated for 45 min with rabbit anti-rat DDT antibodies (6) or non-immune rabbit Ig as a control. Biotinylated goat anti-rabbit antibodies (DAKO A/S, Glostrup, Denmark) were used as secondary reagents and were further detected by ExtrAvidin peroxidase (Sigma, St. Louis, MO, USA) and developed with diaminobenzidine (Saveen Biotech, Malmö. Sweden).

Statistical analysis

Individual changes after UVB exposure were tested by use of the Wilcoxon signed-rank test. Differences were considered to be statistically significant at $P < 0.05$. Spearman's rank-correlation test was used for testing of the association between pairs of variables. The non-parametric test was used because only 10 subjects were studied and it was not obvious that the material had a normal distribution.

Results

In the dopachrome tautomerase assay, fluid of epidermal suction blisters was obtained from 10

healthy subjects. D-dopachrome tautomerase activity, measured in terms of DHI formed from D-dopachrome (DDT activity), was demonstrated in blister fluid from all tested subjects (15–66 nmol/ml × min). All but one of the subjects showed increased activity after three MED of UVB, and the mean value increased from 33.6 to 74.6 (range from −14 to 105) (Fig. 2). This difference is statistically significant ($P = 0.006$).

MIF activity, measured in terms of DHICA formed from D-dopachrome, was also found in blister fluid from all of the subjects (0.5–4.0 nmol/ml × min). Nine out of 10 showed increased values after three MED of UVB, and the mean value increased from 1.59 to 3.83 (range from −0.7 to 6.4) (Fig. 3). This difference is statistically significant ($P = 0.006$).

The person without increase of DDT and MIF after UVB stimulation had the highest initial concentration of DDT as well as of MIF (subject no. 7). Statistical analysis showed a strong correlation between the individual increase in DDT and MIF activities after UVB exposure (correlation coefficient $r = 0.915$) (Fig. 4). Blister fluids from non-irradiated and irradiated sites in one of the subjects were also analysed by Western blot, confirming the presence of DDT and MIF (6).

Immunohistochemical analysis of normal non-irradiated skin revealed a moderate binding of the anti-DDT antibodies all over the epidermis, however, with a tendency to increased binding in the basal layer. Biopsies from normal skin 24 h after irradiation with three MED of UVB showed a stronger staining throughout the epidermis (Fig. 5a,b). Staining with control Ig was negative (data not shown).

Discussion

Exposure to UVB radiation has been shown to induce a release of several cytokines in the skin as well as into the blood (12). MIF was one of the first cytokines to be described, and an increase in MIF concentration in serum after total body exposure to UVB was recently reported by Shimizu et al. (17). They also demonstrated the increased production of MIF by cultured human keratinocytes after UVB irradiation. Interestingly, they registered a biphasic course with an early increase in serum concentration of MIF 1–12 h after irradiation and a late response after 12–48 h. They speculated that the early response was caused by irradiated keratinocytes and the late response was related to other cells. The inflammation they obtained at total body irradiation with four MED of UVB could be strong enough to stimulate MIF production distal from the skin. It is known that MIF is secreted by the pituitary cells after stimulation with pro-inflammatory agents such as lipopolysaccharide (LPS) (9). However, in our study we analysed MIF and DDT in blister fluid after three MED of UVB irradiation of 10 cm^2 on the forearm. This small area should hardly give any systemic stimulation of MIF or DDT production. Although MIF and DDT are small proteins with free diffusion from the blood into the extracellular fluid, we believe that the increase of the proteins in blister fluid studied in our work represents locally produced substances and that any contribution from distal cells can be ignored. Furthermore no D-dopachrome tautomerase activity giving DHI could be measured in normal serum samples (8). The presence of DDT in normal epidermis and its increase after UVB irradiation was further confirmed by immunohistochemical analysis. The staining pattern of DDT with a somewhat more distinct staining in the basal layer of epidermis in non-irradiated skin and throughout epidermis after UVB irradiation mimicked the pattern already described in a similar analysis of MIF (17).

In this work we analysed D-dopachrome tautomerase activity. Because DDT is the only known enzyme that converts D-dopachrome to DHI we think the measurement of this conversion is an appropriate way to determine the DDT concentration. In a similar way the MIF concentration was determined by measuring the D-dopachrome tautomerase activity giving DHICA. No other enzyme is known to give this conversion.

The production of DDT was found to correlate with MIF concentration measured as its enzymatic activity. DDT and MIF have several properties in common. Murine DDT shows 27% amino acid identity with MIF, and foldings and topologies are similar (6, 26). Besides two microbal enzymes (27) MIF and DDT show few similarities to other proteins. In contrast to MIF, DDT is a newly defined protein and its physiological role is not defined. Besides their structural similarities, DDT and MIF both show the same keto-enol tautomeric activity on phenylpyruvate derivatives (6) but give different products when D-dopachrome is the substrate (1).

In this study, D-dopachrome tautomerase activity was demonstrated in blister fluid, and DDT activity (conversion of D-dopachrome to DHI) was seen in all healthy subjects. All but one of these subjects showed increased values of DDT after UVB irradiation, and the mean value was raised approximately twofold in our experimental model. MIF activity (DHICA formed from D-dopachrome) was also found in blister fluids from all the subjects, with all but one showing increased values after three MED of UVB and the mean value also raised approximately twofold. The person who showed no increase of DDT after UVB stimulation was the same person who showed no increase of MIF.

DDT has been demonstrated in human blood cells and liver (5, 8) but no other human studies have been published. In this study we show for the first time the presence of DDT in normal human skin. We also report the increase of DDT in the skin when an inflammation is induced using a model based on UVB irradiation. Its correlation with MIF is of special interest as MIF has been correlated with inflammation in several organs in the body and also in the skin (17). It is supposed to act as a pro-inflammatory cytokine acting together with TNF-α and INF-γ (3). Whether these reactions are due to cytokine properties or enzymatic activity has not been settled for MIF. DDT has up to now been known as an enzyme. Whether or not it also has cytokine properties and glucocorticoid-regulating properties such as those of MIF still has to be shown.

Acknowledgements

This investigation was supported by grants from the Swedish Foundation for Health Care and Allergy Research, the Swedish Council for Work Life Research, the Swedish Cancer Society, the Alfred Österlund Foundation for Scientific Research, and the Edvard Welander Foundation for Scientific Research.

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