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Prevention by α-difluoromethylornithine of skin carcinogenesis and immunosuppression induced by ultraviolet irradiation

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Summary. Administration of α-difluoromethylornithine (DFMO) to mice was found to inhibit both the cutaneous carcinogenesis and the immunosuppression induced by ultraviolet B (UVB) irradiation. BALB/cAnNTacfBR mice were given 1% F_2MeOrn in their drinking water throughout the experiment. After 3 weeks, mice received UVB irradiation consisting of five 30-min exposures per week to banks of six FS40 Westinghouse sunlamps. In the photocarcinogenesis study, mice received a total dose of approximately 1273 kJ m^{-2}. Skin cancer incidence in UV-irradiated mice was 38% 28 weeks after the first UV exposure; DFMO reduced this incidence to 9% ($P=0.025$, log-rank test). Although DFMO has been demonstrated to be chemopreventive of chemical carcinogenesis, this is the first report that it is effective against cancers induced by a physical carcinogen. The immunosuppression induced by UVB irradiation prevents the host from rejecting antigenic, syngeneic UV-induced tumors, which normal mice can reject. The level of immunosuppression in UV-irradiated mice treated with DFMO was measured by a passive-transfer assay. Splenocytes from UV-irradiated mice to naive mice prevented the recipients from rejecting 20/24 UV-induced tumor challenges, whereas splenocytes from UV-irradiated mice treated with DFMO did not prevent recipients from rejecting such challenges (2/24 grew). The difference between these values was significant ($P<0.001$, two-sample test for binomial proportions). Phenotypic analysis of splenocytes used in the passive transfer, using a biotin-avidin-immunoperoxidase technique, revealed that DFMO treatment prevented the reduction of Ia expression normally seen in UV-irradiated mice. Thus, administration of DFMO reduced skin carcinogenesis and immunosuppression induced by UVB irradiation.

Key words: Immunomodulation – Photocarcinogenesis – Chemoprevention – Skin cancer – Mouse

Introduction

Skin cancer is the most frequent of human tumors, accounting for more than 50% of all cancers reported annually in the United States (Scotto et al. 1983). Although skin cancer can be prevented by avoidance of sun exposure, its incidence has been rising steadily, approximately 17% from 1972 to 1978, according to statistics compiled by the National Cancer Institute (Fears and Scotto 1982). Therefore, skin cancer ranks as a significant public health problem.

Murine photocarcinogenesis provides a model in which mudulation of immunosuppression can be studied concomitantly with prevention of primary tumorigenesis. Ultraviolet B (UVB) irradiation induces an immunosuppression that limits the host's capacity to respond immunologically to antigenic, syngeneic tumors induced by UV irradiation or certain chemicals (Gensler 1988; Roberts and Daynes 1980). A number of antigenic tumors have been found to induce an immunosuppression, which prevents their rejection (North and Bursuker 1984; Dye and North 1981; Mukherji et al. 1987; Mullen et al. 1985; Hoon et al. 1987). It may be clinically important, therefore, to find means of preventing or overcoming those types of immunosuppression that prevent tumor rejection.

The requirement for polyamines in cell proliferation (Pegg 1986) suggests that their biosynthetic pathway can serve as a target for tumor prevention. Difluoromethylornithine (DFMO) is an enzyme-activated irreversible inhibitor of ornithine decarboxylase (ODC), which is the first and rate-limiting enzyme in polyamine biosynthesis (Pegg and McCann 1982). In addition to its role in polyamine synthesis, ODC has recently been found to have immunomodulating properties, such as down-regulation

Abbreviations: IL-1, -2 etc. interleukin-1, -2 et; DFMO, α-difluoromethylornithine

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of interleukin-2 production (Flescher et al. 1989; Mihm et al. 1989). Therefore, the induction of cutaneous ODC by UV irradiation may lead to local immunomodulation, which could reduce the host capacity to respond immunologically to UV-induced antigenic skin tumors. On the basis of this rationale, we have tested the capacity of the ODC inhibitor, DFMO, to prevent the immunosuppression and carcinogenesis induced in murine skin by UV irradiation. Immunosuppression was measured by a passive-transfer assay. Influences of DFMO on the phenotypes of splenocytes used in the passive-transfer assay were also evaluated.

Materials and methods

Mice: Seven-week-old specific-pathogen-free female BALB/cAnNTacfBR ($H2^d$) mice were purchased from Taconic Laboratory Animals, Germantown, NY. Animals were maintained in microisolators, under barrier conditions. They were cared for according to the United States Department of Health and Human Services Guidelines for Animal Care. The solid diet consisted of the synthetic AIN76A diet, but contained 100 g/Kg of corn oil.

Administration of DFMO. BALB/c mice received 0 or 1% α-difluoromethylornithine (DFMO) in the drinking water 3 weeks before the onset of UVB radiation treatments and throughout the experiment. The DFMO used in this study was kindly supplied by Merrell Dow Research Institute. The same source of sterilized tap water was supplied to control mice. Both drinking waters were constantly available for the duration of the experiment. The average fluid and DFMO intake was as follows: UV-irradiated mice, 5.5 ± 0.267 ml mouse^{-1} day^{-1} containing 0 mg DFMO mouse^{-1} day^{-1}; UV-irradiated mice + DFMO, 5.4 ± 0.290 ml mouse^{-1} day^{-1} containing 54 mg DFMO mouse^{-1} day^{-1}; unirradiated mice, 5.3 ± 0.223 ml mouse^{-1} day^{-1} containing 0 mg DFMO mouse^{-1} day^{-1}.

Ultraviolet irradiation. After 3 weeks of DFMO administration, mice began UVB (280–320 nm) radiation treatments. They were shaved dorsally each week, and exposed to UV radiation emitted by banks of six FS40 Westinghouse fluorescent sun lamps for 30 min per day, 5 days a week. These lamps emit approximately 80% of their total energy output in the wavelength range of 280–340 nm. The BALB/c mice underwent 12 or 21.5 weeks of intermittent UV irradiation for the passive transfer and the photocarcinogenesis studies, respectively. These treatments resulted in approximate total doses of 711 kJ m^{-2} or 1273 kJ m^{-2}, as measured by a UVX Digital Radiometer with a UVX-31 sensor (Ultraviolet Products Inc., San Gabriel, Calif.).

Passive-transfer assay. The preparation of splenocytes for injection into the lateral tail veins of naive recipients has been described previously (Gensler 1988). In the present experiments, spleens were excised aseptically from 26 mice/group after 12 weeks of UVB irradiation, pooled according to group, and single-cell suspensions were prepared by extrusion of spleen contents into RPMI-1640 medium, with subsequent trituration. Cellular aggregates were allowed to settle in the test-tube, and the supernatant was withdrawn for intravenous infusion via a lateral tail vein into naive normal mice. Cells were transferred into 24 naive mice/group. Within 24 h, 8×10^5 UVM12 (syngeneic, antigenic, UV-induced) tumor cells were injected subcutaneously into the flank of each splenocyte recipient. Tumor growth was assessed weekly.

Phenotypic analysis of splenocytes. An avidin-biotin-immunoperoxidase technique was employed (Grogan et al. 1983; Hsu et al. 1981; Delellis et al. 1979). Aliquots of the splenocyte suspensions (pooled from 26 mice/group) used for the passive transfer were cytocentrifuged onto slides and fixed in acetone. The cells were sequentially incubated at room temperature with a primary monoclonal antibody for a leukocyte differentiation or activation antigen, a biotinylated antibody reactive with the first antibody (TAGO Inc., Burlingame, Calif.) and avidin-biotin-peroxidase complex (Vector Labs, Burlingame, Calif.), each for 25 min. Slides were washed with phosphate-buffered saline after each incubation. The slides were stained in a 3,3′-diaminobenzidine (Sigma Labs, St. Louis, Mo.) and hydrogen peroxide bath and counterstained with methylene green. The specificities and source of the primary antibodies are shown in Table 1. All assays included normal splenocytes on which normal rat serum replaced the primary antibody. All slides were evaluated independently in a blinded fashion by two investigators. Morphologies of cells were determined using preparations stained with hematoxylin and eosin. The cells counted ranged from 600 to 900 sample^{-1} antibody^{-1}.

Table 1. Monoclonal antibodies used in this study

Monoclonal antibody	Hybridoma clone	Specificity	Source[a]
Lyt1	53-7.3	T cell differentiation antigen, murine analog of the CD5 molecule	1
Lyt2	53-6.7	T cytotoxic/suppressor cell, murine analog of the CD8 molecule	1
L3T4	GK 1.5	T helper-inducer cell murine analog of the CD4 molecule	1
IL2R	AMT-13	Interleukin-2-receptor-positive cells	2
Mac1	M1/70	Resident and tumoricidal macrophages	2
Mac2	M3/38	Phagocytic macrophages	2
Ia	M5/114	$Ia^{b,d,P,q,r,k}$	2

[a] 1, Becton Dickinson Company, Mountain Viev, Calif.
2, Boehringer-Mannheim Corporation, Indianapolis, Ind.

Statistical analysis. In the photocarcinogenic studies, the Kaplan-Meier method of survival analysis was used to determine if there was a difference in the proportion of tumor-free mice between the groups of mice. The P value was obtained using the log-rank statistic. In the passive-transfer experiment, the two-sample test for binomial proportions was used to compare the proportion of mice with visible tumors in the DFMO treated versus untreated mice. Differences in the splenic phenotypes were analyzed using the standard t-test for difference in two proportions.

Results

Reduction of photocarcinogenesis by dietary DFMO

The rate of cutaneous tumor formation in UV-irradiated mice is shown in Fig. 1. At 28 weeks after the first UV exposure, the probability of tumor development was 38% in mice fed the control diet, but only 9% in mice that had been given 1% DFMO in their drinking water. The difference in probability of tumor formation between the

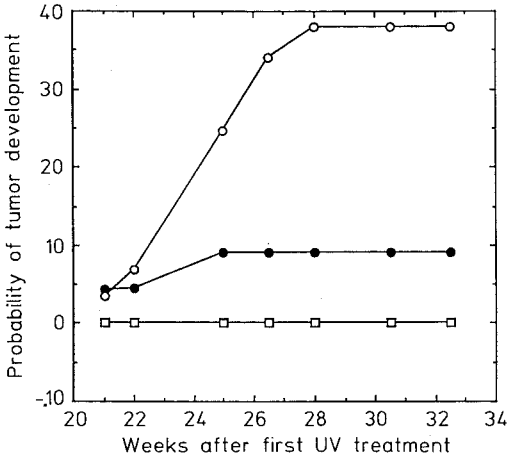

Fig. 1. Rate of tumor development in UVB-irradiated mice treated with α-difluoromethylornithine (DFMO). Groups of 35 mice were fed a basal diet, AIN 76A and given 1% F_2MeOrn in their drinking water throughout the experiment. After 3 weeks of F_2MeOrn, mice began to receive five 30-min UVB radiation treatments per week for 21.5 weeks. The difference in values between UV-irradiated mice with DFMO treatment versus those without was significant ($P=0.025$, log-rank test). ●, DFMO +UV; ○, basal diet +UV; □, basal diet −UV

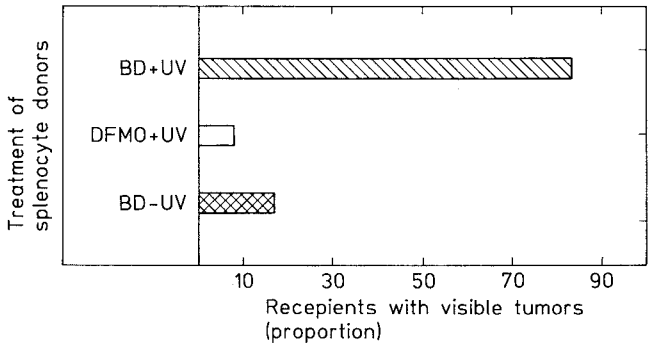

Fig. 2. Growth of UVM12 tumor cells in syngeneic mice after injection of splenocytes from UV-irradiated donors treated with DFMO. Mice were exposed to approximately 711 kJ m^{-2} delivered intermittently over 12 weeks. Splenocytes, pooled from 26 donors/group, were injected intravenously into 24 naive syngeneic recipients/group. Within 24 h, 8×10^5 UVM12 cells were injected subcutaneously into the flank of each splenocyte recipient. Tumor growth or rejection was assessed 3 weeks after implantation. The difference in proportion of tumors that grew between UV-irradiated mice with and without DFMO treatment was significant ($P<0.001$, two-sample test for binomial proportions). *BD*, basal diet; *DFMO*, α-difluoromethylornithine

groups was significant, on the basis of the log-rank test ($P=0.025$).

Reduction of ultraviolet-radiation-induced immunosuppression by dietary DFMO

To determine whether or not dietary DFMO influenced the induction of immunosuppression by UV irradiation, splenocytes from UV-irradiated mice were transferred to naive mice 24 h before the recipients were implanted with antigenic, syngeneic, UV-induced tumor cells. Of the recipients that received splenocytes from UV-irradiated mice, 83% (20/24) bore visible tumors 3–5.5 weeks after tumor injection (Fig. 2). These tumors were all larger than 5 mm^3 and continued to grow until the termination of the experiment 5.5 weeks after challenge. Only 8% (2/24) of the recipients that were injected with splenocytes from UV-irradiated mice treated with 1% DFMO had visible tumors during this time. The difference between these groups was significant, on the basis of the two-sample test for binomial proportions ($P<0.001$).

Phenotypic analysis of splenocytes used in the passive-transfer assay

Aliquots of the splenocyte suspensions used in the passive-transfer assay were reacted with monoclonal antibodies specific for leukocyte differentiation or activation antigens. The proportion of splenic leukocytes that reacted with antibodies to macrophage antigens is presented in Table 2. UVB irradiation of mice resulted in a decrease in the proportion of splenocytes that reacted positively for Ia ($P<0.05$), the major histocompatability complex class II antigen, which is required for effective antigen presentation. DFMO treatment of mice undergoing UVB irradiation prevented this down-regulation of Ia^+ expression on splenocytes, but also led to a decrease in the proportion of $Mac2^+$ splenocytes ($P<0.001$). The proportion of nucleated splenocytes that reacted with antibodies to T cell differentiation antigens is presented in Table 3. Treatment of mice with UVB irradiation resulted in an increase in the proportion of splenocytes that reacted positively for the interleukin-2 receptor, a marker for T cell activation, relative to values obtained in unirradiated controls ($P<0.05$, standard *t*-test for difference in two proportions). The treatment of mice undergoing intermittent UV irradiation with F_2MeOrn did not influence this result. However, the DFMO treatment did yield an increase in the proportion of splenocytes positive for the L3T4 antigen ($P<0.01$)

Table 2. Expression of macrophage or histocompatibility class II antigens in splenocytes from UV-irradiated mice

Donor treatment	Proportion of leukocytes positive for:		
	Mac1[a]	Mac2	Ia
+UV	0.157±0.027[b]	0.111±0.023[c]	0.505±0.051[c]
+UV+DFMO	0.177±0.030	0.051±0.010	0.620±0.048[d]
−UV	0.087±0.016	0.077±0.015	0.576±0.050

[a] Proportion of positive cells ± standard error of the proportion, in pooled splenocytes from 26 donors/group. 600–900 cells antibody^{-1} group^{-1} were analyzed
[b] Different from negative control value, $P<0.001$, standard *t*-test for difference between two proportions
[c] Different from negative control value, $P<0.05$, standard *t*-test for difference between two proportions
[d] Different from UV-irradiated positive control value, $P<0.05$

Table 3. Expression of T lymphocyte antigens on splenocytes from UV-irradiated mice

Donor treatment	Proportion of leukocytes positive for:			
	Lyt1[a]	Lyt2	L3T4	IL-2R
+UV	0.125±0.022	0.082±0.015	0.174±0.029	0.066±0.013[b]
+UV+DFMO	0.126±0.023	0.104±0.019	0.250±0.038[c]	0.056±0.011
−UV	0.114±0.021	0.071±0.014	0.175±0.030	0.034±0.007

[a] Proportion of positive cells ± standard error of the proportion, in pooled splenocytes from 26 donors/group. 600–900 cells antibody^{-1} group^{-1} were analyzed
[b] Different from negative control value, $P<0.05$, standard t-test for difference between two proportions
[c] Different from UV-irradiated positive control value, $P<0.01$, standard t-test for difference between two proportions

Side-effects of chronic DFMO treatment in UV-irradiated mice

The body weights of mice fed 1% DFMO in the drinking water averaged 10% lower than the control UV-irradiated mice, until the last 2 weeks of the experiment, when the DFMO treated mice were found to be 16% lower in weight than the controls (data not shown). On the basis of previous experimental results using this same photocarcinogenesis protocol in our laboratory (unpublished observations), a 10% lower body weight would not account for the reduction in skin cancer by DFMO. In addition to an influence on body weight, DFMO treatment produced hyperactivity in some of the UV-irradiated mice. The hyperactive behavior was characterized by agitated running upon stimulation with noise, e.g., the sound of animal clippers. After 1 month of 1% DFMO treatment, only $^{1}/_{6}$ mice was hyperactive, whereas $^{12}/_{60}$ mice showed this behavior after 3 months of DFMO. Interestingly, chronic administration of DFMO to man has resulted in a reversible loss of hearing acuity, which has been found in up to 54% of patients (Abeloff et al. 1986; Croghan et al. 1988).

Discussion

Treatment of mice with 1% DFMO in the drinking water dramatically reduced the incidence of cutaneous tumors resulting from UVB irradiation. This effect was seen in the total number of tumors formed rather than as a change in latency. Similar protective results have been found with DFMO inhibition of skin tumor promotion by 12-*O*-tetradecanoylphorbol 13-acetate or chrysarobin and with complete carcinogenesis by dimethylbenz[*a*]anthracene (Verma et al. 1986; DiGiovanni et al. 1988). DFMO has been found effective in the prevention of tumorigenesis in a number of organs, e.g. murine colonic tumors induced by 1,2-dimethylhydrazine (Kingsnorth et al. 1983), rat mammary gland carcinoma induced by 1-methyl-1-nitrosourea (Thompson et al. 1985), and rat urinary bladder carcinogenesis induced by *N*-butyl-*N*-(4-hydroxybutyl)nitrosamine (Uchida et al. 1989). Thus, induction of ODC appears to be a widespread requirement for the process of carcinogenesis.

The mechanisms by which DFMO prevents photocarcinogenesis could include inhibition of cell proliferation (Pegg and McCann 1982), inhibition of DNA damage repair in UV-irradiated cells, resulting in loss of replicative capacity of DNA-damaged cells (Snyder and Sunkara 1990), inhibition of angiogenesis (Takigawa et al. 1990), and/or immunomodulation (Flescher et al. 1989; Bowlin et al. 1987, 1990). The finding that ODC down-regulates interleukin-2 (IL-2) production (Flescher et al. 1989; Mihm et al. 1989) indicates that ODC can function as an immunomodulator. DFMO treatment of mice undergoing intermittent UVB irradiation prevented the development of immunosuppression and, therefore, it was acting as an immunomodulator in this system. Phenotypic analysis of aliquots of the splenocytes used in the passive-transfer assay revealed significant alterations in leukocyte surface antigens associated with UV irradiation and DFMO treatment. UVB irradiation of mice led to a reduction in the proportion of Ia^+ splenocytes, even though the proportion of Mac1 and Mac2 splenocytes increased. Previous reports have shown a correlation between the reduction of Ia^+ expression in mouse splenocytes after UV irradiation and a decreased capacity for antigen presentation (Greene et al. 1979). Together, these findings suggest that the lack of Ia expression limits the antigen-presenting capacity of an increased proportion of splenic macrophages. Treatment with DFMO of mice undergoing intermittent UV irradiation prevented the down-regulation of Ia antigen on splenocytes and prevented the development of immunosuppression. The proportion of $L3T4^+$ cells was increased in those UV-irradiated mice that were treated with DFMO. It will be of interest to determine the extent to which these alterations in lymphocyte phenotypes reflect functional changes.

Induction of T-lymphocyte mitogenesis by IL-1, IL-2, and IL-3 has been found to require polyamine biosynthesis (Endo et al. 1988; Bowlin et al. 1986). Suppression of IL-1- or IL-2-induced proliferation by DFMO was entirely overcome by addition of exogenous putrescine. Since putrescine can be endogenously converted to spermidine and spermine by sequential additions of an aminopropyl group, the specificity of any one polyamine with lymphokine-induced mitogenesis remains to be determined. Although DFMO has recently been reported to enhance macrophage tumoricidal activity (Bowlin et al. 1990), little information is presently available concerning

the role of DFMO in immunomodulation during carcinogenesis. Lymphocyte activation by antigens ultimately results in proliferation of functionally mature cells. However, multiple cellular interactions must first occur among the constituents of the immune system. Effective antigen presentation requires a second stimulus in addition to peptide presentation in the context of an MHC class II antigen. The helper T cell that recognizes the antigen must then produce IL-2 in order to stimulate proliferation of mature T cell populations and other cytotoxic effector cells (Balkwill 1989). Although DFMO can inhibit the down-regulation of IL-2 production by ODC, induction of proliferation in lymphocytes by IL-2 requires and stimulates induction of ODC and c-*myc* (Reed 1987; Klinken et al. 1988; Farrar et al. 1988). It is possible that the ability of DFMO to restrict c-*myc* expression (Reed 1987) and thereby limit lymphocyte proliferation could prevent the expansion of T suppressor cells, and prevent UV induction of a significant systemic immunosuppression. Interestingly, there is evidence that DFMO treatment of mice leads to the loss of T cells that negatively regulate, or suppress, erythropoiesis (Sharkis et al. 1983). The injection of putrescine into the mice overcame the erythropoietic effects of DFMO, suggesting that the polyamine biosynthetic pathway was directly involved in this negative regulation.

The present results show that DFMO can significantly prevent cutaneous carcinogenesis and immunosuppression induced by UVB irradiation. Phenotypic analysis of splenocytes revealed that DFMO prevented the reduction of Ia expression commonly found in UV-irradiated mice, and associated with deficient antigen presentation (Greene et al. 1979). These results suggest that immunomodulation by DFMO may contribute to its ability to prevent photocarcinogenesis.

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