Suppression of UV Carcinogenesis by Difluoromethylornithine in Nucleotide Excision Repair-deficient Xpa Knockout Mice

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ABSTRACT

Xeroderma pigmentosum (XP) patients are deficient in nucleotide excision repair (NER) because of mutations in one of the genes coding for NER enzymes. This results predominantly in high frequency of UV-induced skin tumors at an early age; the most severe phenotype is found in patients of complementation group A (XPA). However, in a subset of these XPA patients no skin tumors appear, even at advanced age. Fibroblasts of this subset of patients are not capable of raising UV-induced enhanced reactivation (ER) of viruses and up-regulation of ornithine decarboxylase (ODC). We hypothesized that prevention of ODC induction would protect NER-deficient patients from cancer.

To simulate the situation in XPA patients, we used a hairless XPA knockout mouse model and down-regulated the ODC activity by difluoromethylornithine (DFMO) administered in the drinking water. The DFMO treatment significantly suppressed UV-induced carcinogenesis. In a crossover study, we additionally found that discontinuation of the DFMO treatment resulted in a rapid appearance of skin tumors, up to levels found in mice not treated with DFMO. Late-stage DFMO treatment significantly reduced the number of carcinomas by a factor of 2–3, and it appeared to select for carcinomas with high ODC activity. These results indicate that DFMO suppresses the outgrowth but not the initiation of UV-induced tumors. The DFMO treatment reduced the tumor load but did not offer the XPA knockout mouse full protection against UV carcinogenesis.

INTRODUCTION

XP is a recessive hereditary disorder resulting in a high sensitivity of the skin to sunlight. One of the hallmarks of the disease is an estimated thousand-fold increased susceptibility to skin cancer at sun-exposed areas, resulting in the onset of skin cancer at a very young age. The disease is caused by mutations in one of the genes required for NER (1). XPA-deficient fibroblast strains are defective in NER at various degrees, depending on the XP gene that is affected. However, most of the XP fibroblast strains are still fully capable of raising UV-induced cellular responses, such as the ER. In contrast, a small subset of XP strains does not show ER, regardless of the affected XP gene. Intriguingly, all of these ER-deficient XP strains were derived from XP patients that did not develop skin cancer, although they reached relatively advanced ages (2). By comparing ER-deficient XP cells with ER-proficient XP cells, we were able to show that ODC transcription and ODC activity are not increased by UV irradiation in the ER-deficient XP cells but are in ER-proficient XP (and normal) cells. Thus, a correlation was found between the inability to induce ODC and ER in fibroblasts, and a certain “resistance” against skin carcinogenesis in the patients from whom these fibroblasts were derived (3).

ODC, a key regulator of polyamine biosynthesis, has been used for a long time as a tumor marker, because its activity reflects cell proliferation (4). In recent years evidence has accumulated that ODC itself has oncogenic potential. For example, overexpression of ODC will transform NIH/3T3 cells (5). The ODC gene is a transcriptional target for the well-known proto-oncogene c-myc (6). On the basis of studies using transgenic mice that overexpress ODC in the skin, it has been postulated that ODC might be a proto-oncogene involved specifically in skin carcinogenesis (7, 8). The effects of ODC overexpression can usually be alleviated by specific inhibitors of ODC, such as the ornithine analogue DFMO. In accordance, DFMO has been shown to be moderately effective as an anticancer agent in rodents and humans (9, 10). In a previous study (3), we have successfully used DFMO to completely block the ER response in ER-proficient XP fibroblasts derived from the cancer-prone XP patients. The next obvious question was if DFMO would be able to block enhanced skin cancer susceptibility in XP in vivo, i.e., simulate ER-deficient XP-patients with lower tumor risk. To address this issue, we decided to expose Xpa−/− knockout mice to UVB radiation in the presence or absence of DFMO. Our experiments indicate that DFMO suppresses the promotion stage (outgrowth of tumors), but not the initiation, of skin cancer in Xpa knockout mice.

MATERIALS AND METHODS

Xpa Knockout Mice. Inactivation of the mouse Xpa gene was accomplished by replacing exons 3 and 4 with a neo marker cassette, followed by homologous recombination in embryonic stem cells (11). Hairless Xpa−/− knockout mice were obtained by crossing 129/ola-C57Bl/6 Xpa−/− knockout mice with albino hairless mice (SKH1; Charles River, Sulzfeld, Germany) for two generations. The progeny was genotyped by a PCR assay with tail DNA.

The animals (male and female) with ages ranging from 6 to 9 weeks at the start of the experiments, were housed individually in standard macrolon cages (size: 23 × 13 × 13 cm), standard mice chow (Hope Farms RMB-H) was available ad libitum. Permission for the present experiments were obtained from the ethical commission on animal experiments of the University Medical Center in Utrecht, The Netherlands, as legally required.

UVB Irradiation and DFMO Treatment. For ODC measurements, 6 of 12 Xpa−/− knockout mice received 16 J/m2 UV/day, with a dose rate of 2 J/m2/min, from automatically time-switched Philips TL-12/40W lamps [54% UV output in UVB (280–315 nm) and 46% in UVA (315–400 nm)] for 6 days. Simultaneously, 1% (w/v) DFMO was administered in drinking water to 3 of the irradiated and 3 of the unirradiated mice, whereas the other mice received normal tap water and served as a control mice. DFMO (Flex Oncology, San Antonio, TX) was dissolved in tap water; pH was corrected with sodium hydroxide to the pH of normal drinking water (pH 6). 1% DFMO was chosen, based on a published dose-response study (12). Administered in the drinking water of CD-1 mice, 1% DFMO was found to be the lowest concentration to reach maximum inhibition (94%) of 12-O-tetradecanoylphorbol-13-acetate-induced ODC activity in the epidermis. At 23 h after the last UV irradiation, the 12 mice were sacrificed. Biopsies of the dorsal skin were snap frozen in liquid nitrogen and used for the ODC assay.

In a UV-carcinogenesis experiment, 18 Xpa−/− knockout mice received...
daily UV exposure under the above-mentioned conditions. Simultaneously, 1% (w/v) DFMO was administered in drinking water to 5 of these mice. Fresh DFMO solution was made every 2 weeks and stored in the dark at 4°C. Drinking bottles were refreshed every week. The 13 other animals received normal tap water and served as a control group.

After 14 weeks the UV lamps were switched off. Simultaneously, the DFMO-treated mice were put on normal tap water, whereas 7 mice of the control group were put on the DFMO regimen. Six animals of the control group continued to receive normal tap water, thus serving as a control group throughout the whole experiment. These treatments continued until the end of the experiment.

Tumor Scoring and Sampling. Every week tumors were scored visually by size, location, and tumor type (either benign papilloma, a clearly protruding, wart-like lesion, squamous cell carcinoma, or precursor lesion (Actinic Keratosis), both lesions with lateral well-vascularized growth). Mice developing tumors >4 mm in diameter were sacrificed. Tumors (diameters >2 mm and < 6 mm) were collected and cut in two. One half of each tumor was fixed in PBS-buffered 4% formaldehyde at 4°C for 24 h for pathological analysis, and the other half was snap frozen in liquid nitrogen. Of the frozen tumors, 4 squamous cell carcinomas from the control group and 4 from the late-treated DFMO group were randomly chosen to determine the ODC activity. The fixed tissues were embedded in paraffin, sectioned, and stained with routine H&E staining. Independent analysis of coded slides was performed by the veterinary pathologist (R. B. B.).

ODC Assay. Dorsal whole-skion biopsies and tumors were homogenized in frozen condition using a Microdismembrator U (B. Braun, Mel-Sungen A.G., Germany) for 1 min at 2000 rpm. The tissue debris was dissolved in a total volume of 2 ml ODC buffer ([pH 7.2), 100 µM EDTA, 2 mM dithiotreitol, 40 µM pyridoxalphosphate, 5 mM NaF, 250 µM phenylmethysulfonylfluoride, and 20 mM KH₂PO₄]. Next, ODC activity was quantified by the production of ¹⁴CO₂ from L¹⁴Cornithine, exactly as described previously (13).

RESULTS

DFMO Suppresses ODC Activity in the Skin of Xpa−/− Knockout Mice. Firstly we examined the extent to which DFMO inhibited the ODC activity in hairless Xpa−/− knockout mice. Treating the mice with 1% DFMO (administered in the drinking water) for 6 days led to a significant decrease (P < 0.01; t test on log-values; pooled data) in ODC activity by 46%. The short-term daily UV irradiation did not increase the ODC activity significantly. Results in more detail are given in Table 1.

DFMO Suppresses UV Carcinogenesis. We tested the effect of DFMO on hairless Xpa−/− knockout mice in UV-induced carcinogenesis. Two groups of mice were subjected to daily low-dose UV irradiations of 16 J/m² from TL-12 lamps. This daily dose is less than half of the minimal edema dose of these UV-sensitive Xpa−/− knockout mice. During this chronic irradiation regimen, which lasted for 14 weeks, both the 1% DFMO-administered group (n = 5) as well as the control group that received normal drinking water (n = 13), did not show any acute effects like erythema or edema, as expected. Both groups started to develop skin tumors at about the same time; i.e., DFMO treatment did not significantly influence the latency time of the first tumors in these small groups of Xpa−/− mice (Fig. 1). When considering the squamous cell carcinomas and precursors separately from the papillomas, we found a tendency toward longer latency times for the carcinomas in the DFMO-treated group (P = 0.07; survival analysis; data not shown).

Significant differences between the groups were found at later time points in the mean numbers of tumors per mouse. Significantly lower numbers of tumors per mouse (P < 0.01 at week 14; Mann-Whitney test) were found in the group of DFMO-administered mice (Fig. 2A). This suppression of the number of tumors per mouse was even more significant (P < 0.005; Mann-Whitney test) for squamous cell carcinomas (Fig. 2B), whereas the differences found in the numbers of papillomas per mouse were not significant (Fig. 2C). This might indicate that DFMO predominantly suppresses the outgrowth of squamous cell carcinomas in this experimental setup.

In contrast to the numbers of papillomas per mouse, the rate of growth of the first visible skin tumors, papillomas, showed a tendency toward slower growth when mice were treated with DFMO. On average, the increase in tumor size from 1.0 mm to ≥2 mm took 3.5 ± 0.9 (SE) weeks in the DFMO-treated (n = 4) and 2.3 ± 0.3 (SE) weeks in the control group (n = 7). First tumors, >2 mm, were selected up to week 15.

To check whether inhibition of ODC would lead to growth retardation, mice were weighed at the end of the first 14 weeks of the experiment. DFMO treatment did not significantly affect the body weight [with DFMO: mean, 28.8 ± 1.2 (SE) g; without DFMO: mean, 27.4 ± 0.6 (SE) g].

The 1% DFMO Treatment Does Not Lead to an Overall Regression of UV-induced Tumors in Xpa−/− Mice. To test whether DFMO treatment leads to tumor regression in our model, we started to administer 1% DFMO to 7 randomly chosen tumor-bearing mice from the control group at week 14. Simultaneously, UV irradiation of all of the mice was stopped to slow down additional tumor induction. Compared with the control group that continued to receive normal drinking water, the late-treated DFMO group developed significantly lower numbers of squamous cell carcinomas per mouse (P < 0.05 at week 18; Mann-Whitney test; Fig. 3), but DFMO did not cause a significant regression as measured by an additional lowering of the total number of tumors per mouse [late-treated DFMO group at week 14, mean, 4.3 ± 0.9 (SE); at week 18, mean, 6.0 ± 1.6 (SE)].
Discontinuation of the DFMO Treatment Causes Rapid Tumor Occurrences. We examined the duration of the chemopreventive effect of DFMO after the treatment was stopped. At week 14, Xpa\(^{-/-}\) mice of the DFMO-administered group (\(n = 5\)) were switched to normal tap water and responded with a rapid (21-fold) increase in the average number of tumors per mouse, reaching the level in the control group within 4 weeks after the switch (Fig. 3), [average numbers of squamous cell carcinomas per mouse, 0.8 ± 0.4 (SE) at week 14 versus 16.6 ± 6.9 (SE) at week 18].

Pathology. The clinical diagnosis of the skin tumors was in general confirmed by histopathology on H&E-stained slides of the tumors. Squamous cell carcinomas or precursor lesions were confirmed by the pathologist (37 of 41; 90%) and the 4 dissimilarly diagnosed tumors were classified as papillomas. Clinical diagnosed papillomas were confirmed by the pathologist (13 of 18; 72%); of the 5 dissimilarly diagnosed tumors, 3 were squamous cell carcinomas or precursors, 1 was a keratoacanthom, and 1 was acanthosis.

ODC Activity in Tumors. Gene amplification of ODC in tumors and tumor-derived cell lines has been reported (14–16), resulting in extremely high ODC activities and DFMO independence. To determine whether this could be the case in tumors of the DFMO-treated group, the ODC activity from 4 randomly chosen squamous cell carcinomas of the late-treated DFMO group were measured and compared with those of the control group. Indeed, the average ODC activity of squamous cell carcinomas of the DFMO-treated group was higher, [ODC activity in late-treated DFMO group, 0.60 (95% CI, 0.01–33) nmol/mg protein/hr; ODC activity in control group, 0.22 (95% CI, 0.02–2.4) nmol/mg protein/hr]. The spread in ODC activities in the tumors was substantially higher than that of the normal skin biopsies (see Table 1). In 3 of the 4 squamous cell carcinomas from the DFMO-treated group, extremely high ODC activity was measured.

DISCUSSION

In this paper we tested the hypothesis that inhibition of ODC activity can rescue the phenotype of Xpa knockout mice, i.e., prevent the hypersensitivity to UV carcinogenesis.

DFMO administered p.o. inhibits ODC activity in wild-type and transgenic mice (10, 17–19). In line with these earlier results, we found that 1% DFMO administered to the drinking water inhibited the
ODC activity in our hairless Xpa−/− mouse model. After 6 days of daily UV irradiations we found no significant increase in ODC activity. Remarkably, up-regulation of ODC activity was found to be stronger at 24 h after a single UV irradiation than after six repeated irradiations during 2 weeks (10), indicating an adaptive response.

DFMO treatment did not result in significantly prolonged latency times of the first tumors in the small groups tested, but it did significantly lower the number of tumors per mouse at later time points. This suppression of tumors was not caused by general growth retardation, because the body weights of the animals were still normal after treatment. Previous UV-carcinogenesis experiments (20) showed that Xpa−/− mice contracted their first tumors four times faster than their wild-type littermates. Hence, the 1% DFMO treatment did not fully rescue the Xpa phenotype but did suppress the number of tumors per mouse substantially.

Because ER-deficient XPA patients (unable to induce ODC) have a markedly prolonged latency time when compared with ER-proficient XPA patients (able to induce ODC; Ref. 3), we would have expected a delay in tumor latency time if ODC was completely blocked. The 1% DFMO inhibited the ODC activity in hairless Xpa−/− mice substantially but not completely; the ODC activity was reduced by 57% in the UV-irradiated group (Table 1). Moreover, XPA patients are shielded from sunlight as soon as this condition is diagnosed (21), whereas the Xpa mice in this experiment received daily UV irradiations.

We found clear chemopreventive action of DFMO on carcinomas and precursors but no significant action on papillomas. This is in agreement with UV experiments with wild-type hairless mice in which DFMO suppressed the squamous cell carcinoma more strongly than the papillomas (10). In contrast to the papillomas, most UV-induced squamous cell carcinomas carry point mutations in the p53 gene (22). Loss of normal p53 function can result in a diminished apoptotic response (less sunburn cells; Ref. 23). DFMO treatment can cause an increase in apoptotic cells in skin tumors (24). Thus, DFMO-induced apoptosis could give a growth hindrance to the squamous cell carcinomas but not to the papillomas (which are mainly associated with Ras mutations; Ref. 22). On the other hand, chemically induced papillomas bearing Ras mutations do regress after DFMO treatment of ODC-overexpressed mice (25). Because of the relative small numbers of papillomas in the present UV experiments, it is too premature to draw hard conclusions on the DFMO effect on UV-induced papillomas.

Although late-stage DFMO treatment did not result in an overall regression of squamous cell carcinomas, it did exert a chemopreventive effect on the number of tumors per mouse. Chemopreventive action or moderate tumor regression after DFMO treatment has been reported (10, 18, 19), whereas substantial regression of skin tumors is found with such a treatment in ODC-overexpressed transgenic mice (24, 25). More successful attempts with DFMO in cancer therapy were obtained in combination with e.g., the cyclooxygenase-1 and -2 inhibitor piroxicam (18, 25).

Discontinuation of the DFMO treatment caused rapid tumor occurrences. The 21-fold increase of the average number of tumors per mouse, 4 weeks after the discontinuation, is unlikely to be attributable to newly initiated tumors; it took 18 weeks to reach this number of tumors per mouse in the mice that received normal drinking water. This indicates again that down-regulation of ODC activity by DFMO treatment suppresses tumor promotion/growth (8). To use DFMO successfully as a therapeutic agent, it would appear to be essential to continue the treatment. This supports the new strategy in clinical trials in which DFMO is administered over long periods of time at low doses, which also eliminates side effects like hearing loss (9). Certainly, treatment of XP patients with DFMO could be of use in the clinical management of these patients in addition to the currently used strict regimen of UV avoidance (21).

An extremely high ODC activity was measured in 3 of 4 squamous cell carcinomas of the DFMO-treated group. Judging by the ODC activity in carcinomas of mice on normal drinking water, a minority of carcinomas may resist DFMO treatment by a constitutively highly elevated ODC activity, possibly caused by gene amplification. This phenomenon of ODC gene amplification has been found previously in tumors and tumor-derived cell lines (14–16).

In summary, we were able to inhibit ODC activity by DFMO in Xpa−/− hairless mice, thus partly simulating the situation in ER-deficient XP patients. As observed in these patients, down-regulation of ODC activity leads to suppression of skin carcinogenesis in UV-irradiated Xpa−/− hairless mice. Discontinuation of DFMO treatment led to a rapid increase of carcinomas, indicating that DFMO suppresses the outgrowth of tumors. Thus, DFMO appears to be effective in tumor prevention but hardly effective in the treatment of pre-existing tumors.

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REFERENCES


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