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Accelerated Appearance of Skin Tumors in Hairless Mice by Repeated UV Irradiation with Initial Intense Exposure and Characterization of the Tumors

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Skin tumors were produced on the back of hairless mice, HOS (HR/De), by exposure to ultraviolet B light (UVB, 290–320 nm) with 4 different protocols. The first tumors appeared earlier (in 10 weeks in group I and 7 weeks in group III) when initial intense exposure was given, followed by repeated lower-level exposures, than when the mice were exposed to the repeated UV only (in 16 weeks both in group II and group IV). All mice developed skin tumors earlier in the groups given the repeated UV exposures three times a week than in the groups given the exposures twice a week. Most of the skin tumors produced by the UVB exposure were histologically malignant, being transplantable to nude mice, and the cultured cells grown from the tumors were capable of producing tumors when injected into nude mice. The accelerated development of skin tumors by initial intense exposure and short intervals of repeated exposure observed in this study may have implications for humans who expose themselves to intense sunbathing and UV tanning (burning) by fluorescent sun lamps.

Key words: UV — Skin tumor — Hairless mouse

Sunlight is the main etiological factor in causing human skin cancers,[1] and the frequency of skin cancer has been increasing in recent years.[2,3] The peak wavelengths of the action spectrum for the carcinogenesis fall into the ultraviolet B (UVB), 290–320 nm, range.[4] Increase in the biologically effective UV radiation doses due to stratospheric ozone depletion,[5] prolonged exposure to sunlight of aged people, and changes in peoples' life style, such as recreational sun exposure, may account for the increase of skin cancer in recent years. Repeated severe sunburns or intense sun exposure in a short period have been suggested to be risk factors for melanoma[6–9] and nonmelanoma[5,10] skin cancers, based on epidemiological studies. These repeated or intense exposures to sunlight might have accelerated the appearance of the skin cancers and increased the incidence.

The purpose of this study was to find conditions of UV exposure which may accelerate the appearance of skin tumors by means of animal experiments. Although there have been several reports[11–14] on UV-induced skin tumors in hairless mice, none of them attempted to shorten the appearance time of the skin tumors by using various protocols of UV exposure. We irradiated hairless mice (not athymic), with or without intense initial UV exposure, with short (3 times/week) or longer (2 times/week) intervals between repeated exposures and compared the tumor development. The UV exposures for both initial intense exposure and the repeated exposures were the maximum that could be applied without causing severe sunburn resulting in necrotic lesions. Skin tumor development presented in this report should therefore correspond the shortest time to the first tumor appearance, as well as to 100% incidence in the exposed mice. Characteristics of the UV-induced tumors were investigated histologically, and cells were cultured from the tumors for transplantation studies.

Abbreviations: MED, minimum erythema dose; SCC, squamous cell carcinoma; UVB, ultraviolet B light.

MATERIALS AND METHODS

Mice Specific-pathogen-free hairless albino mice of the inbred strain HOS (HR/De) were supplied by Hoshino Experimental Animal Farm (Saitama). The strain HOS is a derivative of HRA/SKH-1 originally obtained from the Skin and Cancer Hospital, Temple University, Philadelphia, PA, USA. Nude mice were supplied by Shizuoka Agricultural Cooperative Association for Laboratory Animals (Shizuoka). They were given chlorinated water *ad libitum* and autoclaved mouse chow. Room illumination was on an automated cycle of 12-h light, 12-h dark, and room temperature was maintained at 22–25°C.

UV exposure The UV light source was a bank of 6 Toshiba FL20SE fluorescent sunlamps (dose rate of 6.3 J/m^2/s) encompassing the wavelength range of 280 to 360 nm with a peak at 305 nm. Flux intensity was measured with a UVR-305/365D digital radiometer (Tokyo Kogaku Kikai KK, Tokyo). Four mice were housed in a cage, and three cages were placed on a shelf

25 cm below the lamps during the exposure, and cages were rotated to provide even illumination along the shelf. Exposure of mice to UV was started when the mice were 7–8 weeks old. There were four groups of mice, each group containing 14 animals. Equal numbers of males and females were employed. Details of the procedure for the exposure are given in Table I. For group I, mice were exposed to 1.8×10^4 J/m^2 of UV twice, one day apart, followed by repeated exposures of 3430 J/m^2 each, 2 or 3 times a week for 21 weeks. For group III, after the initial intense exposure as in group I, the repeated exposures were started one month later. For groups II and IV, mice received only the repeated UV exposures two and three times a week, respectively, for about 20 weeks.

Measurement of the cumulative tumor incidence Tumor formation was monitored by palpation and observation, and the day of tumor detection was recorded according to the Kaplan-Meier procedure, excluding dead mice without tumors, according to Hoel and Walburg.[15]

Tumor transplantation to nude mice Each tumor was removed and cut into small pieces (about 1–2 mm in diameter), and 5–6 pieces were transplanted to a nude mouse subcutaneously in each side of the flank with a trocar. Growth of the implanted tumors in nude mice was monitored for 180 days.

Histological examination Tumors were excised, including surrounding normal tissue, fixed in 10% neutral formalin and embedded in paraffin. Sections (5 μm) were stained with hematoxylin and eosin (HE), and examined histologically.

Culture of tumor cells Small pieces were excised from 27 tumors without visible necrosis or infection, and were immersed in culture medium containing antibiotics for 30–60 min, 2 or 3 times. They were minced into 0.5–1 mm^3 pieces with sharp scalpels, and were placed in plastic 25 cm^2 flasks with a small amount of Dulbecco's modified Eagle's minimum essential medium (Nissui, Tokyo) supplemented with 15% fetal calf serum (HyClone Laboratory Inc., Logan, UT). When the cells reached confluency, they were transferred to 75 cm^2 flasks to be used for experiments.

Killing effect of UV on the tumor cells Appropriate numbers of cells were inoculated into 6-cm dishes and incubated for 18 h. Cells were washed with phosphate-buffered saline and irradiated with UVC from germicidal lamps (Toshiba GL-10). Cells were further incubated in medium containing 15% fetal calf serum with a medium change twice a week until colony formation. After fixing and staining, colonies (50 cells or more) were scored.

Inoculation of cultured tumor cells into nude mice After establishment of the tumor cell lines, 10^7 cells were suspended in 0.2 ml of phosphate-buffered saline and injected into a nude mouse subcutaneously in each side of the flank.

RESULTS

Prevalence of tumor development In all groups, all mice developed skin tumors, which appeared only in the areas exposed to UV. Tumors more than 3 mm in diameter were scored, and cumulative tumor prevalance, percent of mice with tumors, was plotted as a function of time (weeks) in Fig. 1. Cumulative UV fluence at the first tumor appearance is shown in Table I. In group I with the initial intense exposure, the first skin tumor appeared 10 weeks after the initial UV exposure, with the cumulative fluence of 9.6×10^4 J/m^2. Prevalence of tumor development gradually increased to 50% in the 22nd week and reached 100% by the 32nd week. In group II, the first

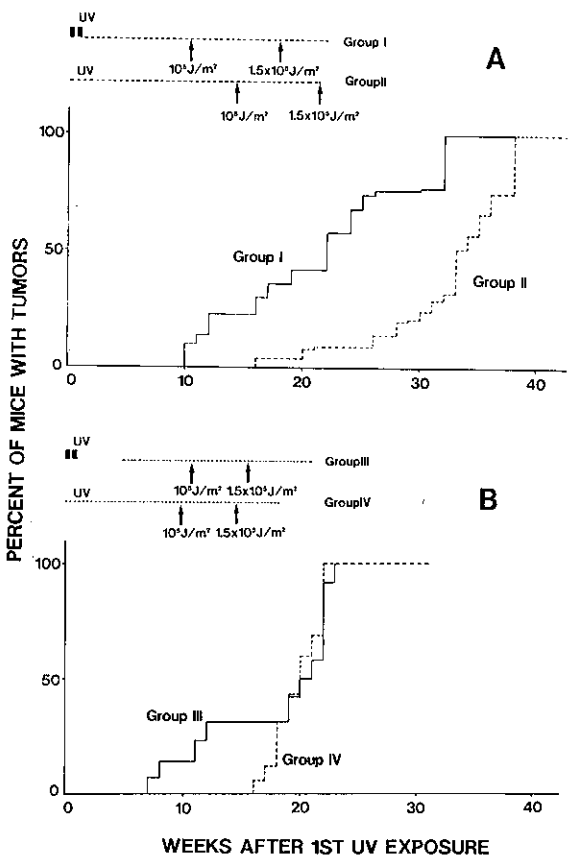

Fig. 1. Cumulative skin tumor prevalence in UV-exposed hairless mice with 4 different procedures. Intense exposures (1.8×10^4, twice) are indicated by closed boxes. Total cumulative doses of 10^5 and 1.5×10^5 J/m^2 are indicated by arrows. (A) Cumulative skin tumor prevalence in mice of groups I and II. Repeated exposures (3430 J/m^2/exposure), twice per week, are indicated by broken lines. (B) Cumulative skin tumors prevalence in mice of groups III and IV. Repeated exposures (3430 J/m^2/exposure), three times per week, are indicated by dotted lines.

Table I. Protocols of UV Exposure and UV Fluence

Group	Initial intense exposure (kJ/m^2)	Total number of repeated exposures[a] (exposures/week)	Cumulative UV fluence at the 1st tumor development (kJ/m^2)	Total UV fluence (kJ/m^2)
I	36 (18×2)	43 (2)	96	180
II		43 (2)	110	150
III	36 (18×2)	48 (3)	46	200
IV		57 (3)	170	200

a) 3430 J/m^2/exposure.

Table II. Histological Types of Tumors Produced by UV Exposure

Group	No. of mice	No. of tumors	No. of tumors per mouse	Experimental period[a] (week)	No. examined histologically (%)	No. of carcinoma	mixed type	papilloma	sarcoma
I	12	22	1.8	32	19	2	3	2	12
					(100)	(10.5)[b]	(15.8)	(10.5)	(63.2)
II	12	25	2.1	38	21	3	5	0	13
					(100)	(14.3)	(23.8)		(61.9)
Subtotal	24	47	2.0		40	5	8	2	25
					(100)	(12.5)	(20)	(5)	(62.5)
III	13	18	1.4	23	16	6	7	1	2
					(100)	(37.5)	(44)	(6)	(12.5)
IV	16	49	3.1	22	49	19	12	0	18
					(100)	(38.8)	(24.5)		(36.7)
Subtotal	29	67	2.3		65	25	19	1	20
					(100)	(38.5)	(29.2)	(1.5)	(30.8)
Total	53	114	2.2		105	30	27	3	45
					(100)	(28.6)	(25.7)	(2.9)	(42.9)

a) Experimental period after which tumor histological analysis was conducted.
b) The numbers in parentheses indicate the percentages.

tumor appeared in the 16th week with the cumulative fluence of 1.1×10^5 J/m^2 and it took 38 weeks to reach 100% prevalence. In group III, a mouse had a visible tumor as early as the 7th week. This mouse had received only 5.0×10^4 J/m^2 before initial tumor appearance. Prevalance of mice with tumors reached 100% in the 23rd week for group III and in the 22nd week for group IV, much earlier than for groups I and II. The times of first tumor appearance in groups III and IV week approximately the same as for groups I and II, respectively. No tumors had appeared in 20 unirradiated mice, 5 mice to each group, after 1 year and three months.

Histological classification of UV-induced tumors It was hard to classify histological types because there were many tumors which showed malignant lesion of both epidermal and dermal components and we made some simplifications according to De Gruijl *et al.*[13] Namely, mixed forms of tumors were categorized according to the dominant aspect. However, some tumors (25.7%) included both dermal and epidermal elements in the same section almost equally, and they were classified as mixed type after Blum[4] and Kripke.[16] Histological types of the tumors are presented in Table II. A total of 114 tumors developed, and 105 of them were examined histologically by conventional hematoxylin-eosin staining. Most of the tumors produced were malignant, as represented by a squamous cell carcinoma (SCC) (Fig. 2A) and a sarcoma (Fig. 2B). In both types, the nuclei are round and large, mitoses are present and the grade of malignancy is high. Sarcomas were most frequently found (42.9%),

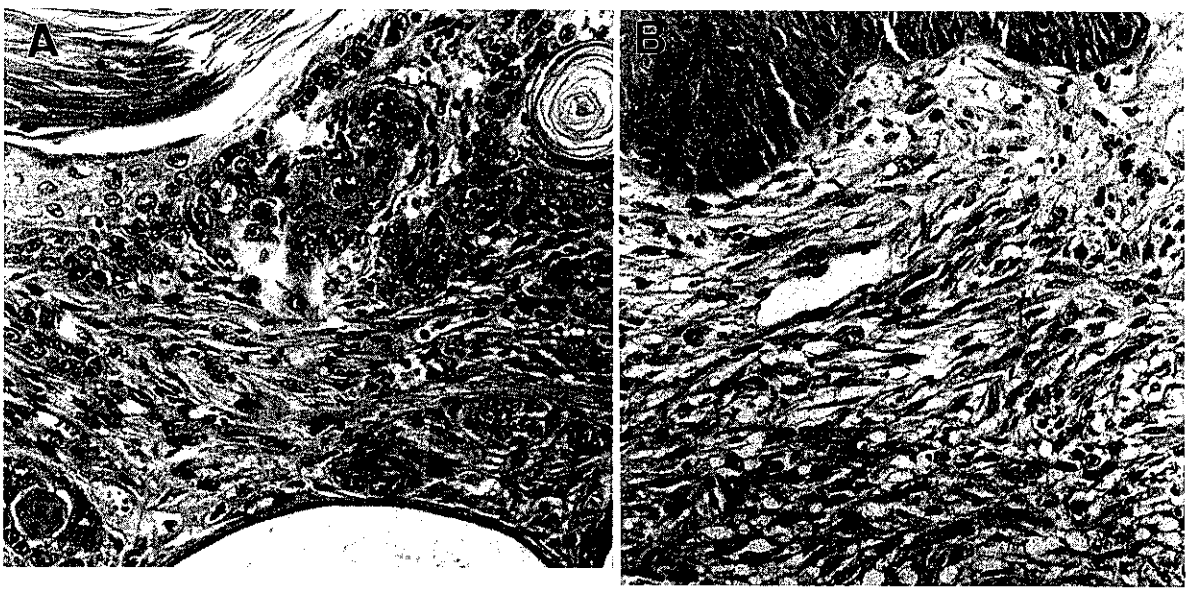

Fig. 2. Representative histology of UV-induced skin tumors in the hairless mice. (A) Well differentiated SCC with horn pearls. (B) Sarcoma consisting of fibroblastic cells with atypical nuclei, arranged in a storiform pattern. (original magnification; ×200)

and SCC accounted for 28.6% of the tumors. Though there was no difference in the histology of the tumors produced by UV exposure with and without the initial intense exposure, frequencies of the histological types between groups I and II vs. III and IV were considerably different, as shown in Table II.

Transplantability of the UV-induced tumors Approximately 88% (72/82) of the tumors were transplantable to athymic nude mice. It took only 2 weeks for the most rapidly growing tumors to become more than 10 mm in diameter and more than 3 months for the most slowly growing tumors to reach that size. Most of the transplanted tumors became larger than 10 mm in diameter within one month. Two papillomas, 3 mixed tumors and 5 SCCs were not transplantable. There was no difference in transplantability among tumors induced by the 4 protocols of UV exposure.

Establishment of cells from UV-induced tumor and their characteristics Primary cultured cells were grown from 26 tumors and have been passaged for more than 1 year. All cultured cells from the tumors were round in shape and readily floated up before confluency, representing typical characteristics of transformed cells, while the cells grown from the normal skin of the tumor-bearing mice were spindle shaped, and quite different from the tumor cells in morphology and growth characteristics (data not shown). Cells from some tumors had a crisis period before becoming established, though cells from most of the tumors grew very rapidly without any crisis.

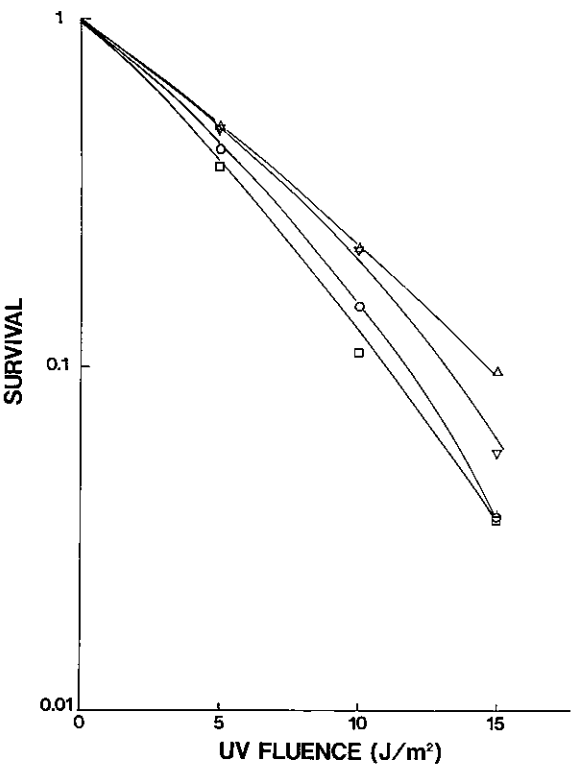

Fig. 3. Cell survival curves after UV exposure of UV-induced tumor cels (□), non-tumor cells (△), cells of an unirradiated region from the same mouse (▽) and cells from the sham-irradiated mice (○).

The extents of UV sensitivity of these tumor cells, non-tumor cells from unirradiated regions and non-tumor cells from UV-irradiated regions were similar, in terms of colony-forming ability (Fig. 3). Most of the tumor cells and non-tumor cells not shown in this figure gave similar results. All tumor-originated cell strains had similar UV sensitivity irrespective of exposure protocol. All cell lines except one grown from a papilloma were capable of producing tumors when injected into nude mice. Normal skin fibroblasts originating from the non-exposed mice were not capable of producing tumors within 3 months after injection into nude mice.

DISCUSSION

There is a clear difference in the course of tumor development between A (groups I and II) and B (groups III and IV) in Fig. 1. In particular, in group IV, all tumors developed within a period of 5 weeks, from the 17th to the 22nd weeks after the initiation of UV exposures. The UV fluence and the frequency of repeated UV exposure used in group IV were the maximum applicable to the hairless mice, since the mice developed acute sunburn with subsequent dermal ulceration and necrosis when the UV doses and frequency of exposures were higher than those used in group IV. The initial exposure of 3.6×10^4 J/m^2 (1.8×10^4 J/m^2, approximately 10 times the minimal erythema dose (MED),[17] twice, one day apart) was the maximum intense UV dose that the hairless mice could tolerate without death due to acute sunburn. From our preliminary experiment, we considered that severe acute sunburn might occur in the case of repeated exposure at 3 times/week starting just after initial intense exposure, although we did not confirm this experimentally. So, an interval of 28 days was set between the initial intense exposure and the beginning of the repeated exposures in group III. With such maximum exposures to UV, all mice in groups III and IV developed skin tumors within 23 and 22 weeks, respectively. The difference in exposure protocols between groups III and IV, with and without the initial intense exposures, caused no change in the required duration for 100% tumor development.

The initial intense exposure, 1.8×10^4 J/m^2, twice, appeared to have shortened the period until the first skin tumor development in groups I and III. Cumulative UV fluences at the appearance of the first tumor were much smaller in groups I and III than in groups II and IV (Table I). The major cause of the early onset of skin tumors in groups I and III, in comparison with groups II and IV, respectively, should be the initial intense exposure to UV. Urbach reported that daily doses attenuated over an 8.3 h period are not as effective in producing tumors as are the same daily doses delivered in 0.083 h (5 min).[5] Our results support this, because of the early onset of skin tumor in groups I and III with the intense initial exposures. One possible explanation is that the intense exposure was more effective than the prolonged exposure, presumably due to more efficient repair of damage in the latter case. Although the removal of pyrimidine dimers in mouse cells was reported to reach 99% within 48 h,[18] the tumorigenic effects of UV could remain in the form of misrepaired pyrimidine dimers or base changes other than pyrimidine dimers.[19] This could explain the effect of the initial intense exposure, which might have yielded unrepaired DNA damage or misrepaired damage. Another explanation is that the initial intense exposure caused rather severe acute sunburn and the wound-healing process could have caused the accelerated development of the tumors, as suggested by Hsu *et al.*[12] Other factors such as natural killer (NK) activity may also be involved in the effect of UV. Our results suggest that total accumulated dose may be less important than the frequency of repeated irradiation or the initial intense exposure, at least as regards the time of appearance of the first tumor (compare tumor appearance at 10^5 J/m^2 in Fig. 1).

The initial intense exposure did not significantly accelerate the course of tumor development to reach 100% prevalence, as shown in Fig. 1. The tumor appearance in group III (at 7 weeks) was the earliest among the 4 groups. As in group I, the initial intense exposure should have accelerated the first tumor development. However, the times required to produce 100% incidence were the same in groups III and IV. This could be due to the promoting effect of UV. The number of the repeated UV exposures (48 in group III and 57 in group IV) could be important, as suggested by Ootsuyama and Tanooka in gamma-ray induced skin tumors in mice.[20]

The UV sensitivities of cell strains originating from UV-induced tumors were all similar, irrespective of exposure protocol. Cells from non-tumor areas exposed to UV and unexposed areas of mouse skin were also similar to those from the tumor (Fig. 3). So, development of skin tumors cannot be explained by any difference in DNA repair capacity in tumor and non-tumor cells. It should be noted that UV-induced tumor cells of the same strains used in this study, showed widely differing sensitivity to alkylating agents,[21] but did not show much difference in UV sensitivity.

Although skin tumors produced by 7,12-dimethylbenz-[*a*]anthracene and 12-O-tetradecanoylphorbol-13-acetate were predominantly papillomas,[22] most UV-induced tumors in our and other studies were histologically malignant, most of the tumors were transplantable and almost all cell lines cultured from the tumors were capable of producing tumors when injected into nude mice, irrespective of experimental group. This could be due to the progression effect of UV, despite differences in the strains

of mice used, or to the duration between tumor development and histological examination. In our study, sarcoma was predominant, while in most studies on UV carcinogenesis in hairless mice, SCCs were most often produced when mice were irradiated daily. Blum suggested that with longer intervals between exposures, development of sarcomas was more frequent than carcinomas in hairy mice.[4] From our data in Table II, the same conclusion can be drawn for hairless mice. The difference in UV lamps (Toshiba in this study and Westinghouse in other reports) might affect the histology of tumor production, but no evidence is available. Morrison *et al.* showed that some UV radiation-induced tumors, which resembled fibrosarcomas in having a spindle appearance upon light microscopic examination, appeared to originate from the epidermis by means of immunoperoxidase examination using anti-keratin antibody, and also by electron microscopic observation.[23] Recently the term spindle-cell carcinoma has become common.[24] The category of mixed type proposed by Blum[4] and Kripke[16] also suggests the presence of neoplasms difficult to diagnose pathologically by light microscopy. Our mixed type could have included spindle-cell carcinoma since we did not confirm our light microscopic determination by additional methods, but the difference between the two types of the cells designated as carcinoma and sarcoma in Table II was clear, and we tentatively concluded that the proportions of different histological types are different in groups I-II and groups III-IV.

The daily exposure of 3430 J/m^2 in the repeated exposures was approximately twice the MED for the hairless mice. Human exposures at such dose levels often take place during sun-bathing and sporting events. Intense exposure as high as 10 times MED may not be usual, except for cases of severe acute sunburn. Our results strongly suggest that people should avoid intense sun exposure and repeated exposures at short intervals. Since the habits and life style of the Japanese population have changed recently, with a tendency to favor intense sun bathing over a short period, skin tumors might develop earlier in life than before, and an increase in the incidence of skin cancer[3] in the future may be anticipated.

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