A-4.1 Carcinogenic Effects of UVB Irradiation on Skin

Contact Person Yasunobu Aoki
Section Chief
Environmental Health Sciences Division, National Institute for Environmental Studies, Environmental Agency
16-2 Onogawa, Tsukuba, Ibaraki 305 Japan
Tel: +81-298-50-2390, Fax: +81-298-50-2588
E-mail: ybaoki@nies.go.jp

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The objective of this study are to elucidate the carcinogenic process of UV-B in order to obtain fundamental minformation on the risk of human health by exposure to UV-B.

1. Mutations in mammlian cells caused by exposure to UV-B

Introduction

Depletion of stratospheric ozone is suspected to cause the increase in irradiation of UV-B, especially around 300 nm, on the surface of earth. Increased irradiation of UV-B will lead to the elevation of mutation frequency in human skin cells, one of whose consequence is the expected increse in skin cancer incidence. In order to assess the effect of increased UV-B on the cancer incidence, we determine the wave-length dependency of relative lethality and relative mutagenicity by UV-B irradiation using our newly-developed assay system to detect the mutation in mammalian cells.

Materials and methods

A retroviral vector (LTK-15) carring both forward (neo) and backward (herpes simplex virus thymidine kinase of HSV-HK gene) selection markers was constructed as a substrate for mutation assay in mammalian cells. The cells infected with this virus are first selected with G418, mutagenized and then selected with the anti-herpes drug acyclovir (ACV). Since HSV-TK, but not host TK, is capable of converting ACV to a toxic metabolite, cells retaining the intact HSV-TK gene fail to survive, while the cells carrying a mutated HSV-TK gene or which have lost the gene can form colonies in the presence of ACV, making it possible to detect the gene defect in a positive manner (1). We prepared a pool of rat fibroblast cells (CREF) infected with this virus (LTK15/CREF cells) and irradiated them increasing doses of ultraviolet light at 290, 300 and 320 nm by Biotronics

monochrome ultraviolet irradiation system (Vilber Lourmat, France), or at 254 nm by GL15 germicidal lamp (Toshiba, Japan). The mumber of suviving colonies and ACV-resistant colonies were counted after incubation for 1 week and 10-14 days, respectively. Total DNA was extracted from some of the ACV/G418 doubly resistent clones and the HSV-TK gene was amplifed by the PCR method. The DNA sequence of HSV-TK gene form the ACV resistant clone was determined by the cycle sequence method.

Results and Discussion

The relative lethality and the relative mutagenicity of the LTK15/CREF cells by UV-B irradiation were similar to those of E. coli (2) at 290 nm and 300 nm, but were one order higher than those of E. coli at 320 nm. Tandem double mutations and multiple mutations were caused by UV-irradiation at 320 nm, while these mutations were rarely observed in the cells which were irradiated at 300 nm.

2. Gene expression and squamous cell carcinoma caused by ultraviolet ray irradiation

Introduction

It has been established that ultraviolet (UV) ray irradiation causes overexpression and mutation of various oncogenes and suppressor genes in cultured cells. However, only a limited number of reports are available with regard to the overexpression and mutation of genes in the squamous cell carcinoma caused by UV ray irradiation in vivo (3-6). In the present study, we have studied the effects of UV B irradiation on the expression and mutation of p53 gene and those of other oncogenes in the squamous cell carcinoma of mice.

Materials and Methods

Hairless mice (5 weeks old, female) were exposed to UVB once or repeatedly at the specified doses. Skin specimens were collected at specified times and used in Northern blot analysis for oncogenes, and in Western blot analysis for p53 proteins. We have analyzed point mutations in Exons 5 to 8 by the use of PCR-SSCP method and determined the position by cycle sequencing method.

Results and Discussion

When mice were singly exposed to UVB at a dose of 3 kJ/m2 and 15kJ/m2, p53 protein was found mainly in the nucleus 24 hours after the irradiation 2 times and 5 times higher over control, respectively. Skin specimens collected hour 16 after the irradiation had apoptotic cells as examined by HE staining. A continuous exposure to UVB at a dose of 2 kJ/m2 up to 10 weeks, caused

papilloma (solar keratosis) as early as 10 weeks after the start of the irradiation, followed by the appearance of squamous cell carcinoma by week 25. These observations were confirmed by histopathological examinations. One third of the mice had the tumor region, the size of which was larger than 3 mm in diameter. In these tumor tissues, it was found that the expression of c-myc, c fos and c-H-ras genes was 2 - 2.5 times higher than control at week 25. The p53 gene product was found in the cytoplasm by Western blot analysis suggesting the occurrence of the mutation of p53.

Next, we analyzed to characterize the mutated p53 gene in Exon 5, 6, 7 and 8 in which the mutation frequency was found high. Among 23 tumor tissues, 11 tissues showed point mutations by PCR-SSCP method in the Exon 5,7 and 8 but not in Exon 6. As summarized in Table 1, there are conversions from C to A, A to G, C to T, C to G in codons in these three Exons.

The present result showed a positive association of the induction of apoptosis with that of mutated p53 protein. As has been reported earlier for UVC and ionizing radiation (7), it is thought that in UVB irradiated cells, wild type p53 protein has a role to terminate the cell cycle at the G1 phase, or to cause apoptosis via Bcl-2/Bax machinery in irreversibly damaged cells, which protects the genome DNA from mutation that is threatening to life.

It has been already established that there are hot spots with frequent mutation in human skin cancer, and that the mutation is characterized as conversion from C to T, which can be explained by the thymine dimer formation. Kress et al. (3) found the similar observation in UVB-caused mouse skin tumor. Our present result was consistent with their earlier observation, but also showed the presence of A to G conversion which has not been reported earlier in solar keratosis in humans, skin cancers in mice and humans including Xeroderma pigmentosum patients (3,5,6).

The point mutation by UVB irradiation is thought to have significant effect on the protein structure and function. It is not clear why the mutation was mainly found in the DNA-binding domain of p53 protein, but is speculated that the mutated p53 will not be able to regulate other genes, which may lead to tumorigenesis.

Table 1 Summary of mutations found in p53 gene of mouse skin tumor.

Exon 5

No	Codon	Sequence	Base change	Amino acid change
4	176	cccac**C**atgag	C ---> A	His --->Arg
15	141	tgtgc**A**gttg	A ---> G	Gln --->Arg
24	161	tctaca**A**gaag	A ---> G	Lys ---> Arg

Exon 7

No	Codon	Sequence	Base change	Amino acid change
17	232	ctaca**A**gtac	A ---> G	Lys ---> Arg
	236	gtgta**A**tagct	A ---> G	Asn ---> Ser
	238	gctc**C**tgca	C ---> T	Ser ---> Ser
	243	gcat**G**aacc	G ---> A	Met ---> Ile
18	236	tgta**A**tagc	A ---> G	Asn ---> Ser
	238	gctc**C**tgca	C ---> T	Ser ---> Ser
	243	gcat**G**aacc	G ---> A	Met ---> Ile
24	236	tgta**A**tagc	A ---> G	Asn ---> Ser
	238	gctc**C**tgca	C ---> T	Ser ---> Ser
	243	gcat**G**aacc	G ---> A	Met ---> Ile

Exon 8

No	Codon	Sequence	Base change	Amino acid change
8	275	gcctgcc**CT**gggag	CT ---> TC	Pro ---> Leu
12	275	gcctgc**C**ctggg	C ---> T	Pro ---> Ser
15	270	ggtt**C**gtgtt	C ---> T	Arg ---> Cys
19	270	ggtt**C**gtgtt	C ---> T	Arg ---> Cys
25	270	ggtt**C**gtgtt	C ---> G	Arg ---> Cys

3. Effect of UVB irradiation on immune competent cells

Introduction

The suppression of delayed-type hypersensitivity and of immunity against certain bacteria or virus by UVB irradiation may be related to the induction of skin tumours and infections. As for the mechanisms of UVB-induced immunosuppression, the impairment of skin immune system such as Langerhans cells and keratinocytes and the loss of systemic immune reactivity are caused by UVB irradiation. Although interleukin 10 (IL-10) and tumor necrosis factor-α (TNF-α) released from immune competent cells may have essential roles, the effects of UVB on the production of other cytokines related to immunosuppression are not clearly understood. We investigated *in vivo* and *in vitro* effects of UVB on interaction of lymphocytes with antigen presenting cells (APC).

Materials and Methods

Male BALB/c, WBB6F1+/+, WBB6F1w/w^v mice (6 - 9 wk old), purchased from Clea Japan Inc., were used in all experiments.

In *in vitro* experiment, BALB/c mice were instilled with ovalbumin (OA) 3 times at an interval of 3 weeks. One week after the last instillation, cervical lymph nodes and spleen were collected from the mice. Cell suspensions of pooled lymph nodes and spleens were obtained by gentle teasing through a stainless-steel mesh. After cell suspensions had been washed 3 times, viable cells were counted. Cervical lymph node cells and APC which were prepared by mitomycin C treatment of spleen cells were cultured with OA. Cultured OA specific T cells or APC were irradiated with different doses of 300nm UVB, as measured by a Biotronic monochrome ultraviolet irradiation system (Vilber Lourmat, France). The sunlamp emitted radiation in the UVB region, and its light was cut off except at 300 nm. UV-irradiated or nonirradiated T cells were cultured with nonirradiated or irradiated APC in the presence of OA for 48 h and the culture supernatants were collected.

In *in vivo* experiment, mice were lightly anesthetized with pentobarbital sodium and were removed the dorsal hair with electric clippers. The UVB dose received by mice was 2KJ/m^2 at 300nm. Immediately after UVB irradiation, mice were instilled with OA intranasally. After the same treatment was repeated 3 times at an interval of 3 weeks, spleens and blood were collected and spleen cells were cultured with OA.

The levels of interleukin 4 (IL-4), IL-10 and interferon-γ (IFN-γ) in the culture supernatants were measured using mouse ELISA kit (Endogen, MA).

Results and Discussion

In the regulation of antibody production, Th1 and Th2 type helper T cells play an important roles. IFN-γ released from Th1 type T cells suppresses Th2 type T cell functions, but IL-4 released from Th2 type T cells increases IgE antibody production and suppresses Th1 type T cell

functions.

In *in vitro* experiments, the levels of IL-4 and IL-10 in culture supernatants of 140J/m^2 irradiated T cells and nonirradiated APC significantly decreased compared to that in nonirradiated T cells and APC. No difference in IFN-γ production was observed in the culture supernatants between irradiated or nonirradiated T cells with nonirradiated APC. However, culture supernatants in 140J/m^2 irradiated APC and nonirradiated T cells increased the levels of IL-4 (Fig.1) and IL-10 production showed normal levels. Our present results indicate that direct irradiation on T cells reduced the activity of cytokine production, but the irradiation on APC increased the IL-4 production from co-cultured T cells.

In *in vivo* experiments, to investigate the cytokine production from spleen cells in UVB irradiated mice intranasally instilled with OA, spleen cells were isolated and were cultured with OA. The levels of IL-4 in culture supernatants of spleen cells from irradiated WBB6F1 mice were the same with that in nonirradiated (control) mice. However, the levels of IFN-γ in culture supernatants of spleen cells from irradiated mice significantly decreased compared to that in control mice (Fig.2). OA specific IgE antibody titters in mice irradiated by UVB were lower than those of control mice. Particularly, UVB irradiation induced marked suppression of anti-OA IgE antibody production in WBB6F1 mice. These results suggest that UVB irradiation and intranasal instillation of OA in mice may modulate *in vitro* antigen-stimulated cytokine production from spleen cells with a consequent decrease in IgE antibody production. Because of no different effect of UVB on mast cell deficient (WBB6F1 w/w^v) and normal (WBB6F1 +/+) mice, the effect of UVB on mast cell functions is not important.

Fig. 1
IL-4 production in culture supernatants of irradiated APC and normal T cells

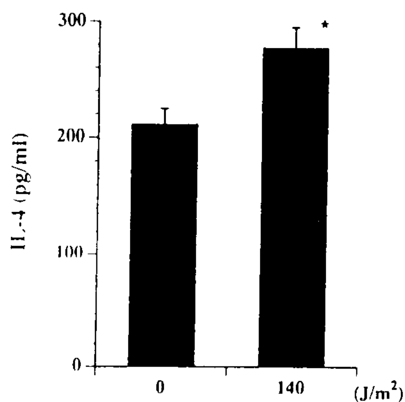
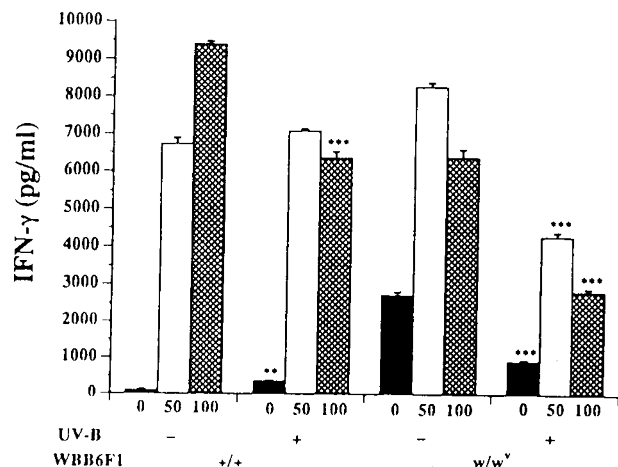


Fig. 2
IFN-γ production in culture supernatants of spleen cells from UVB irradiated WBB6F1 mice



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