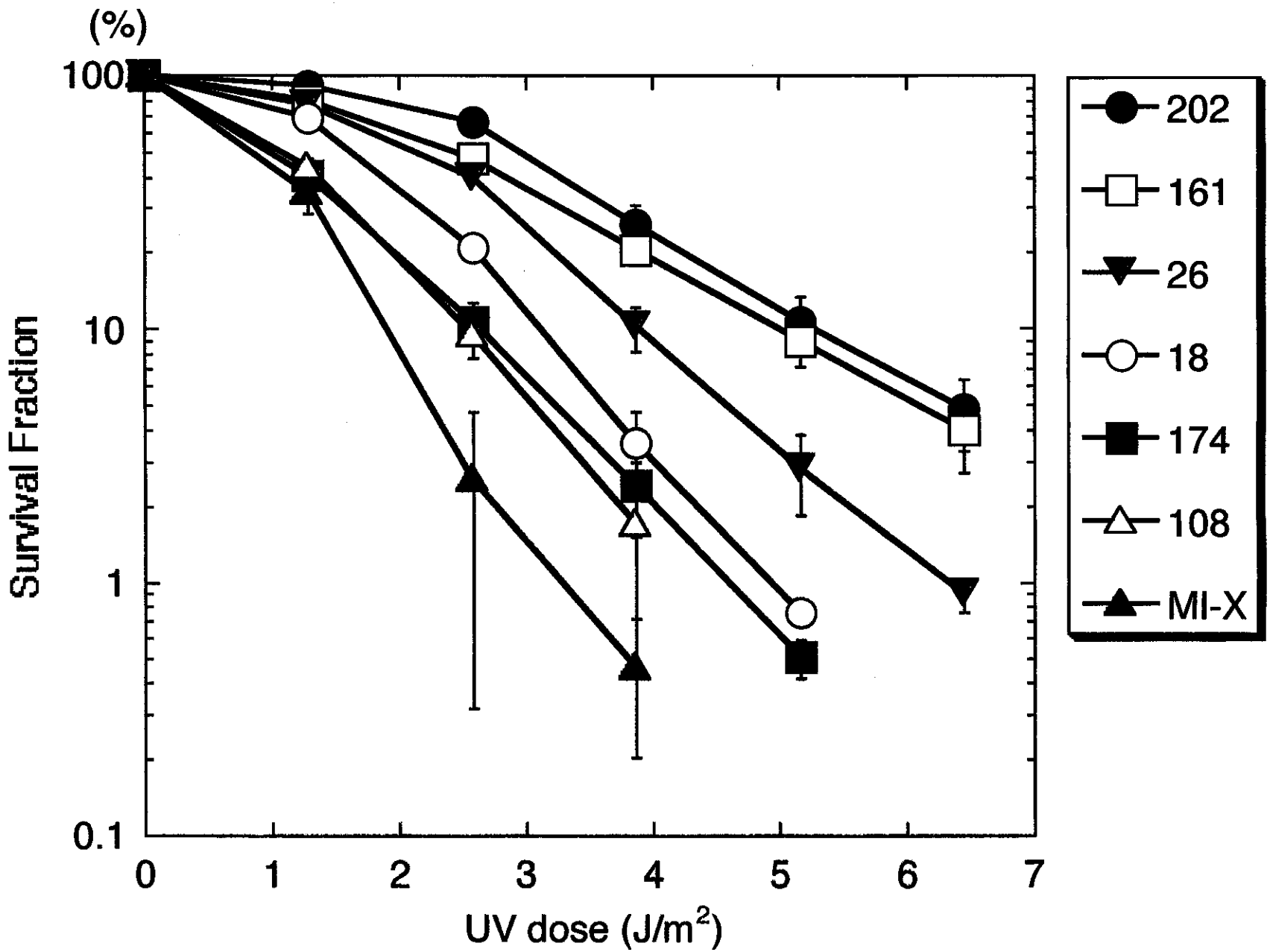
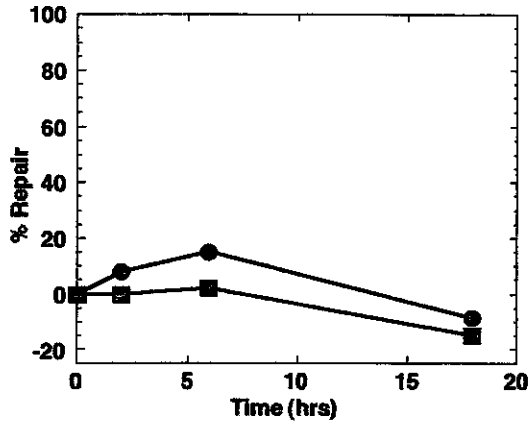
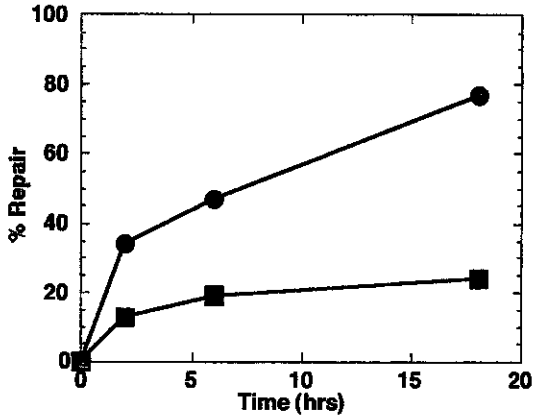

(%)
100
10
1
0.1
Survival Fraction
0
1
2
3
4
5
6
7
UV dose (J/m2)
202
161
26
18
174
108
MI-X

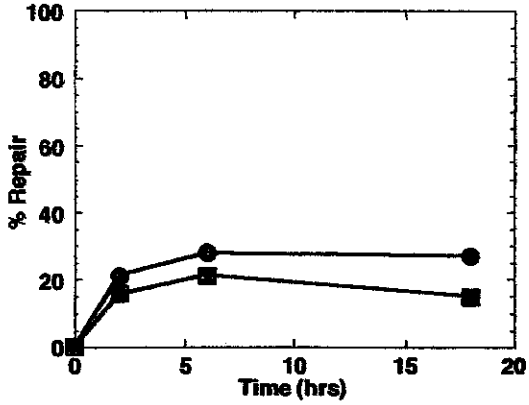
MI-X : XPA(-/-) fibroblast



202 : XPA(+/+) fibroblast



18 : XPA(-/-) skin cancer cell line



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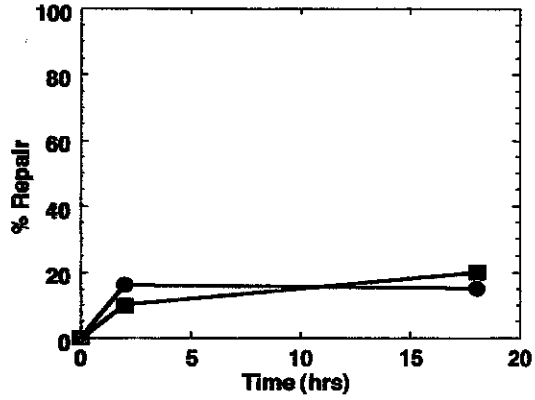
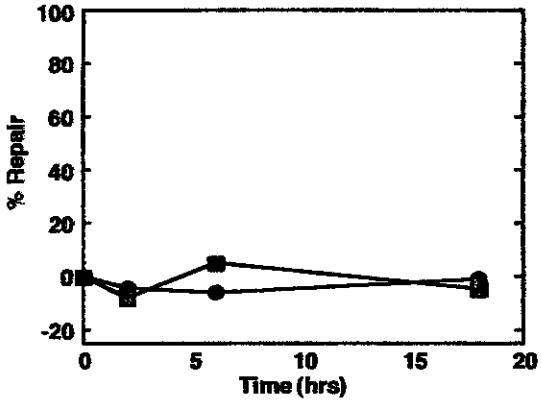
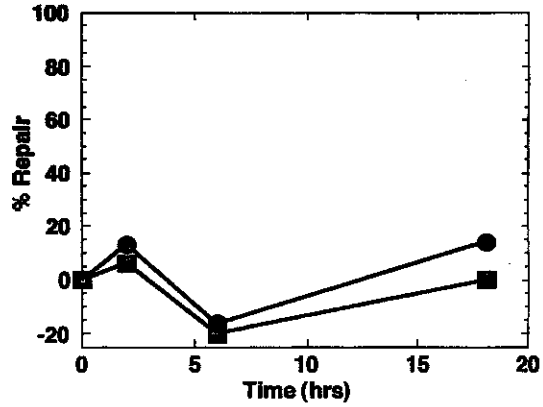


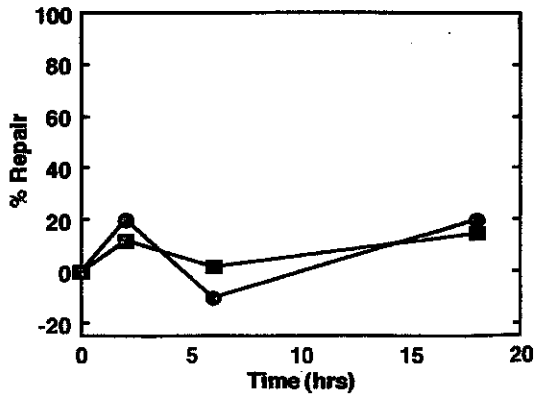
26 : XPA(-/-) skin cancer cell line

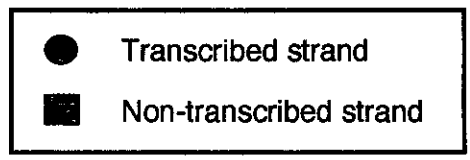


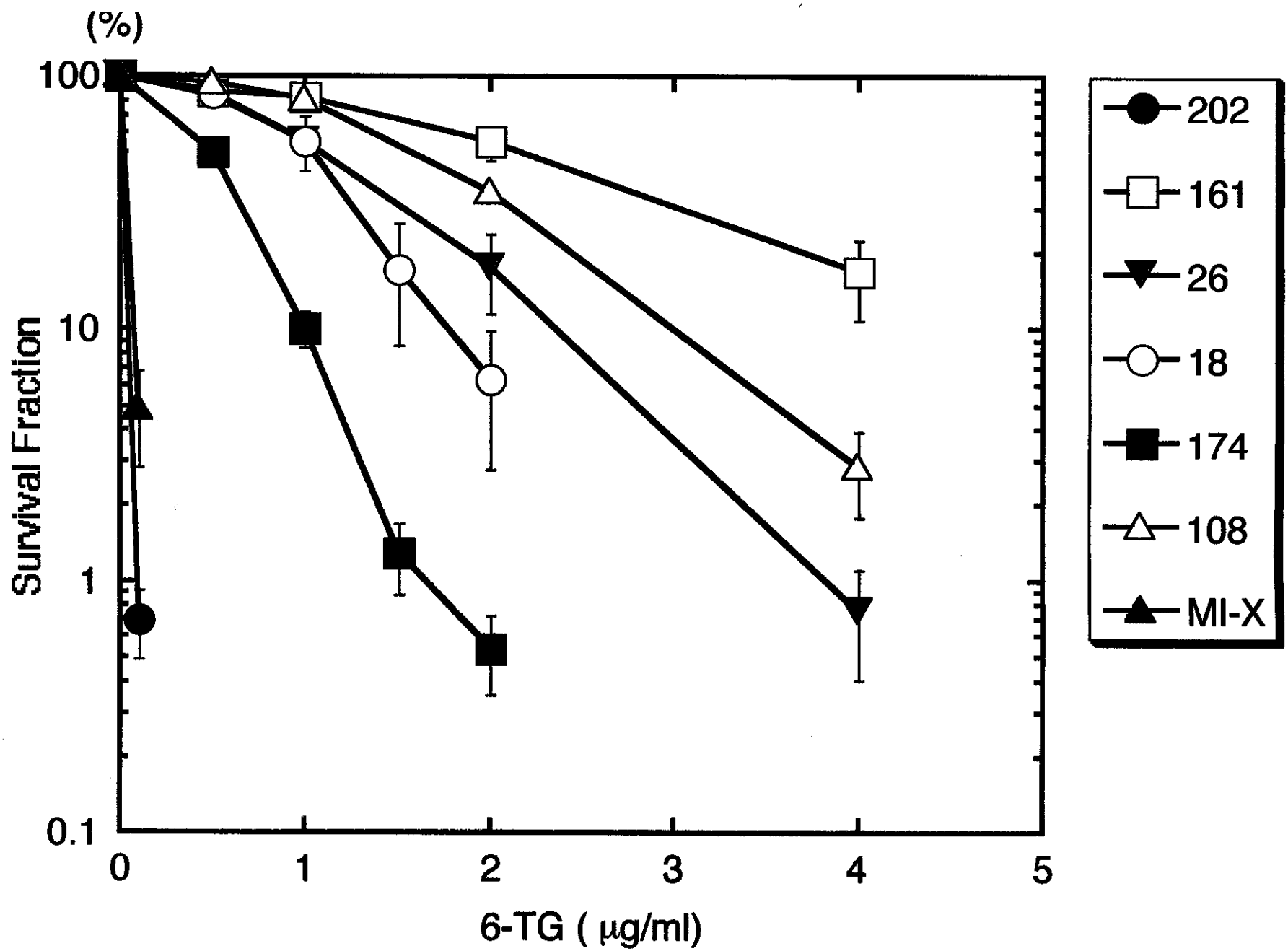
174 : XPA(-/-) skin cancer cell line

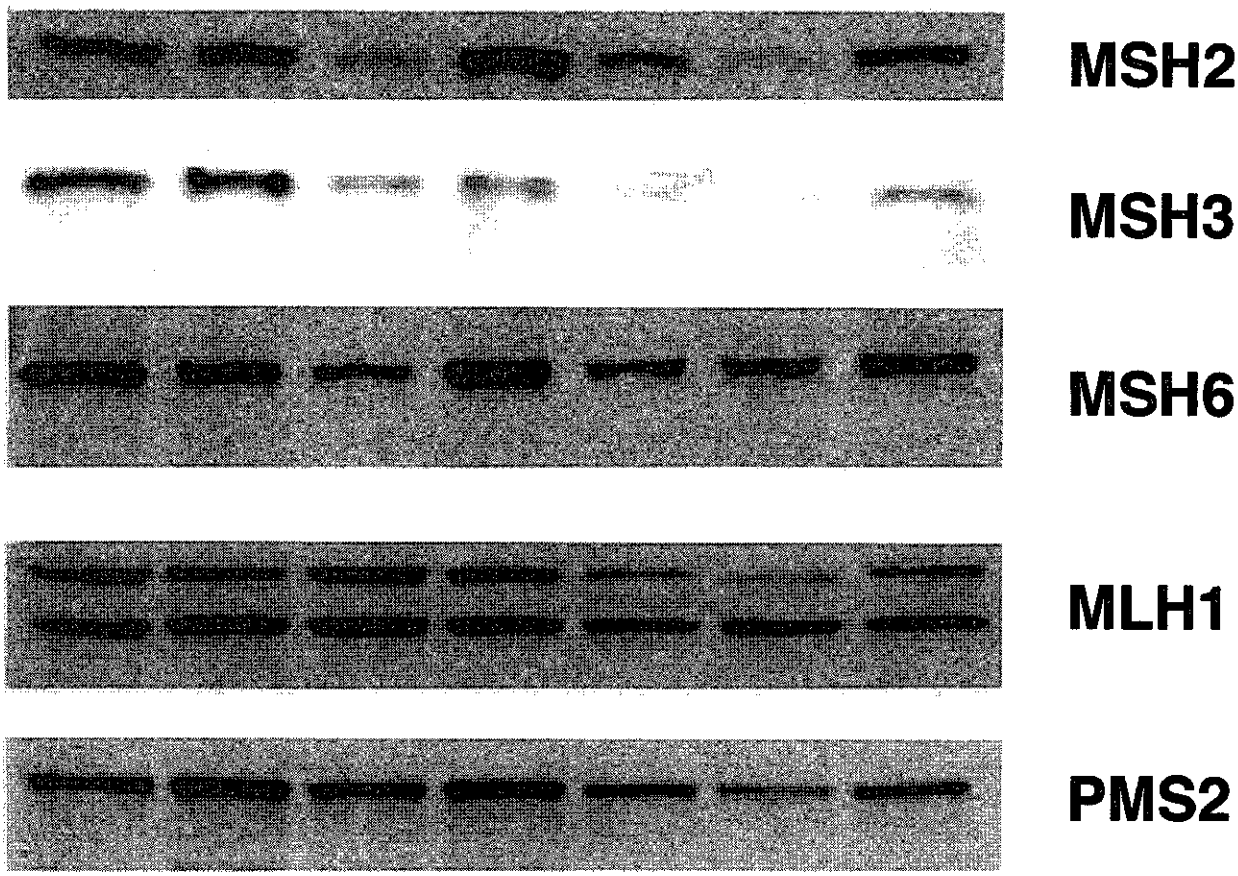


161 : XPA(-/-) skin cancer cell line

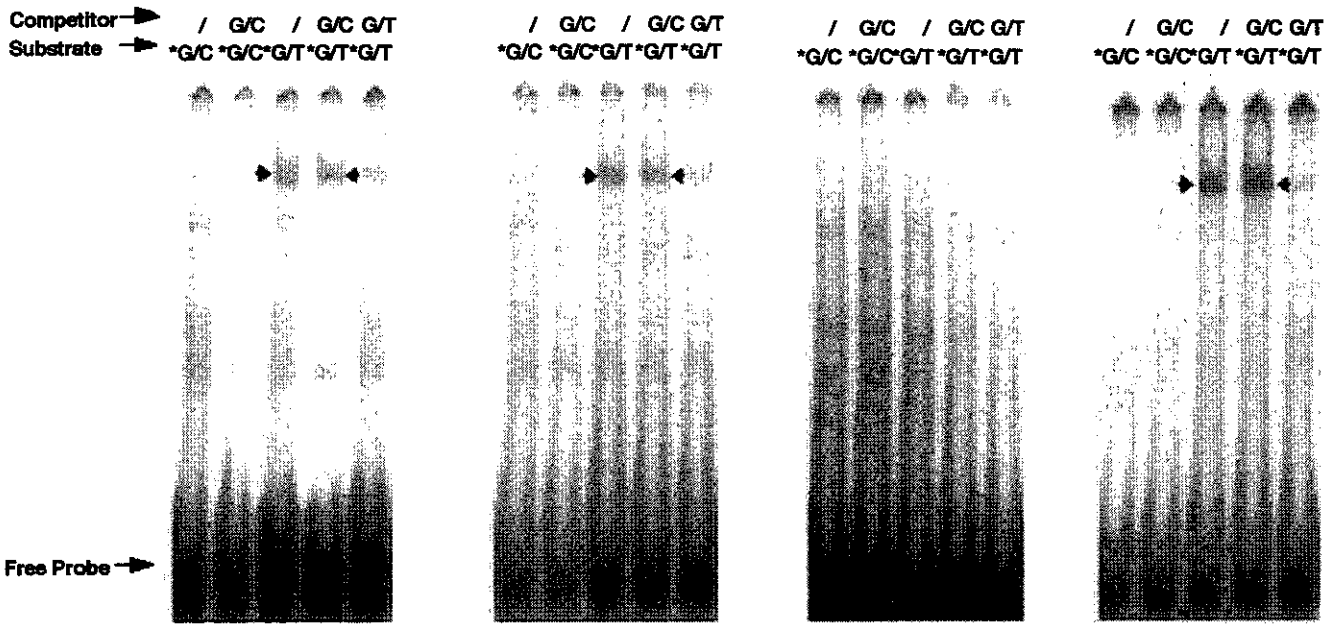




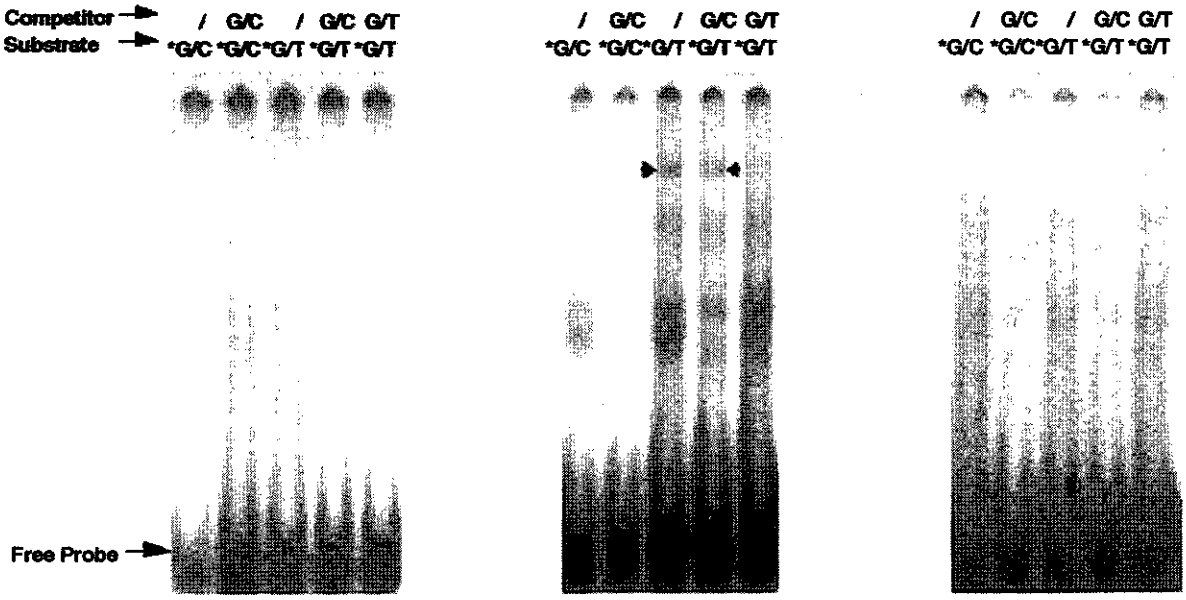

(%)
100
10
1
0.1
Survival Fraction
0
1
2
3
4
5
6-TG (μg/ml)
202
161
26
18
174
108
MI-X

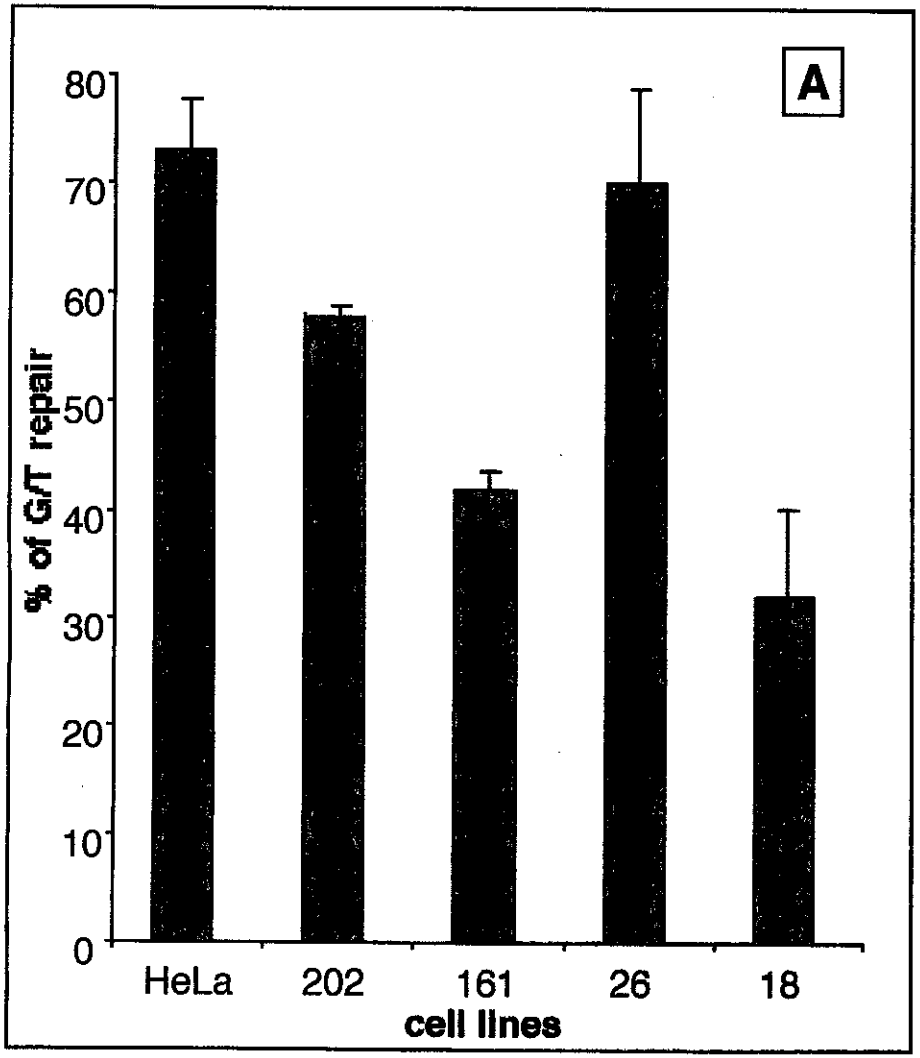

202 MI-X 161 26 18 174 108
MSH2
MSH3
MSH6
MLH1
PMS2

202 MI-X 161 26



18 174 108




A
80
70
60
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40
30
20
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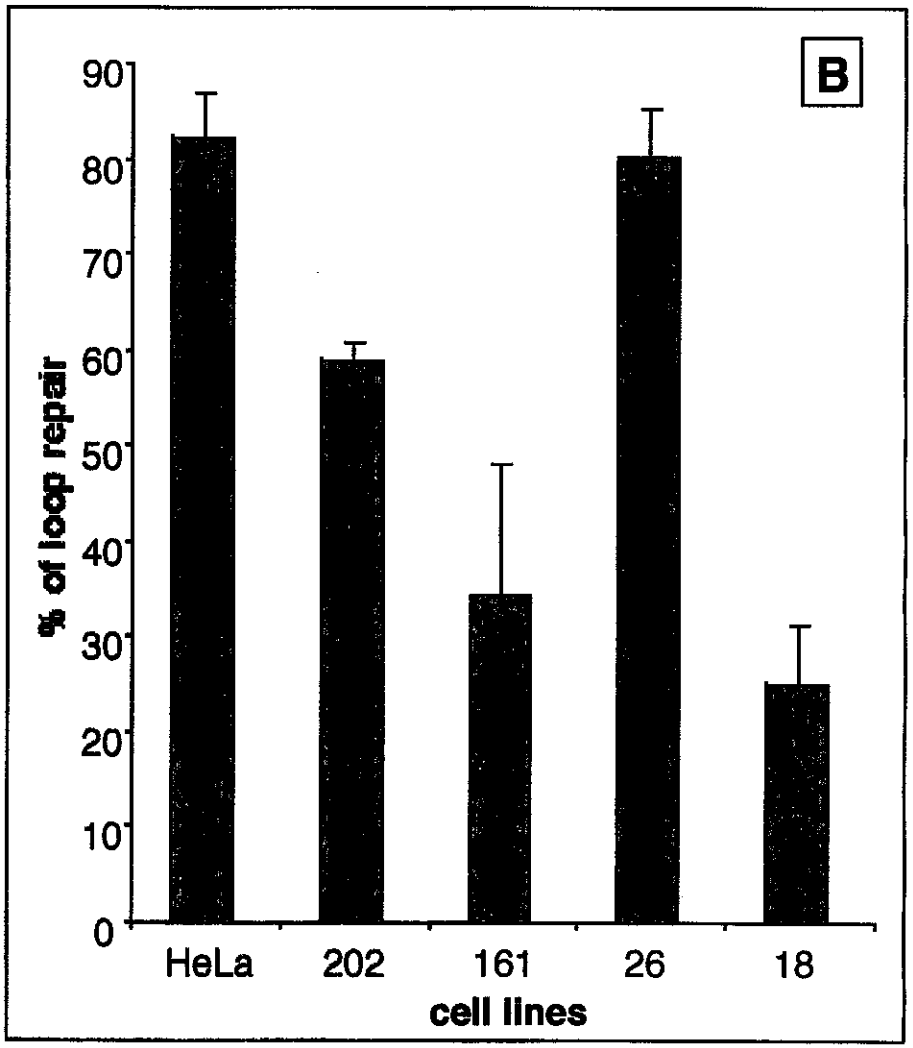
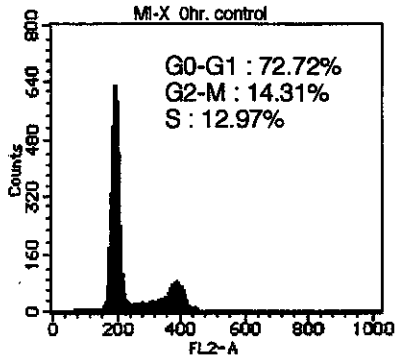
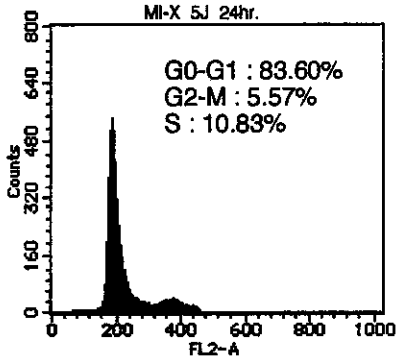
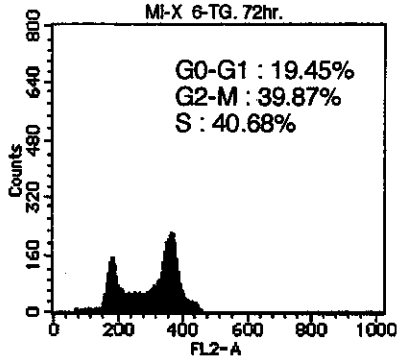
0
% of G/T repair
HeLa
202
161
26
18
cell lines


B
90
80
70
60
50
40
30
20
10
0
% of loop repair
HeLa
202
161
26
18
cell lines

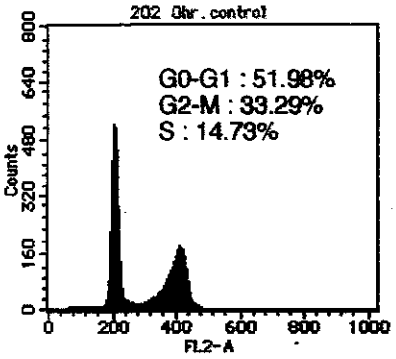
MI-X : XPA-/- fibroblast

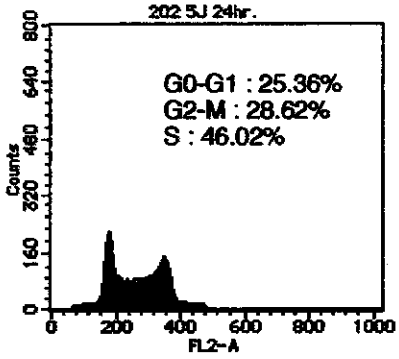


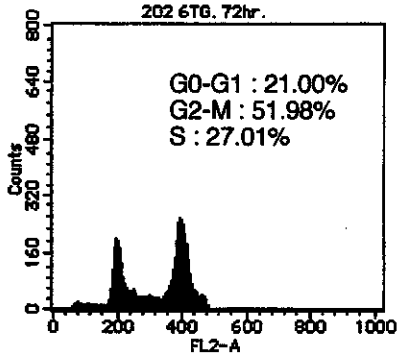




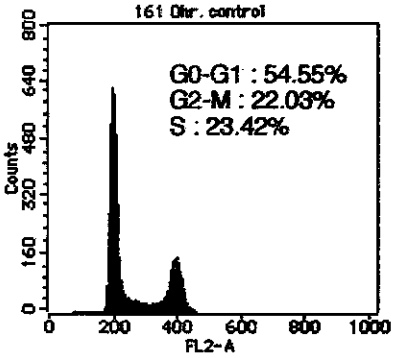
202 : XPA+/+ fibroblast

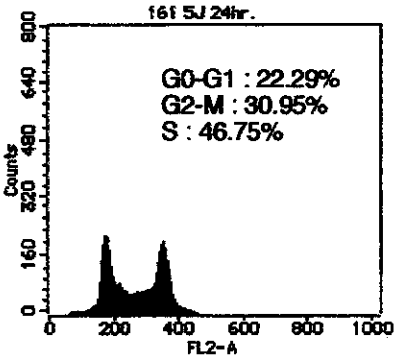


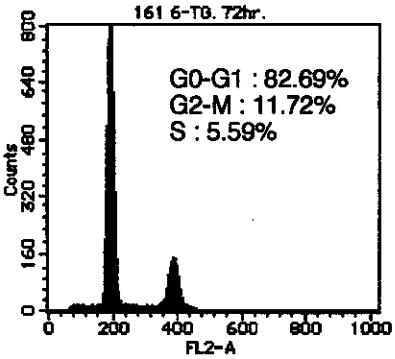




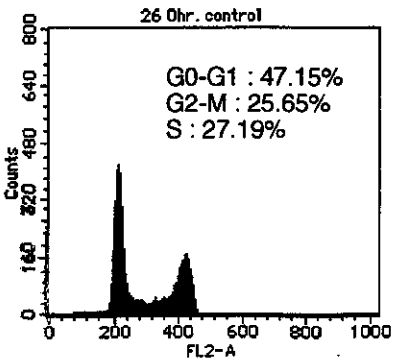
161 : XPA-/- cancer cell line

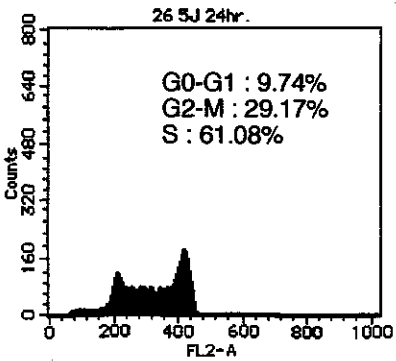


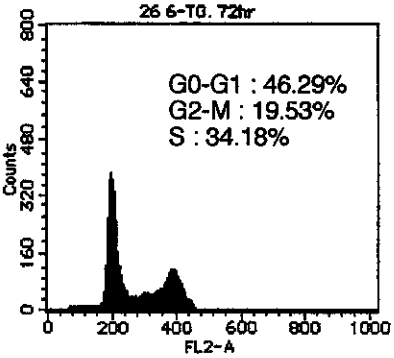




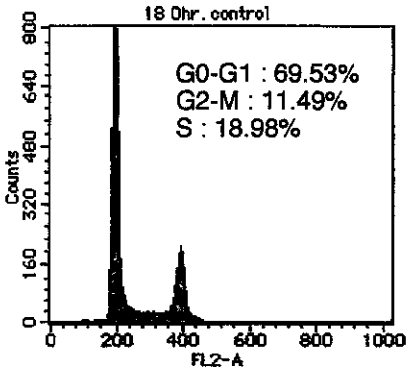
26 : XPA-/- cancer cell line

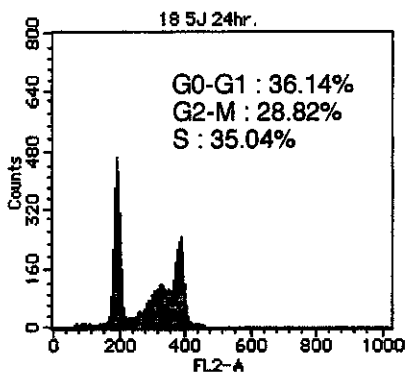


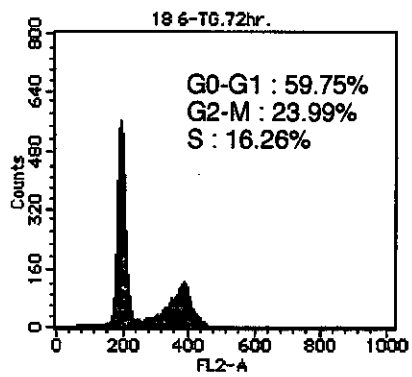




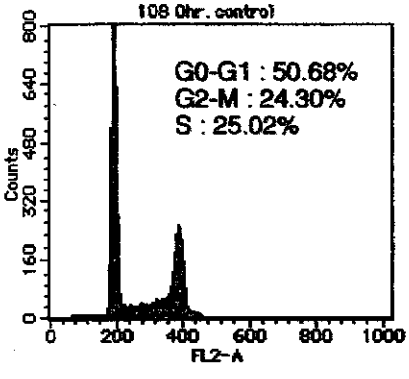
18 : XPA-/- cancer cell line

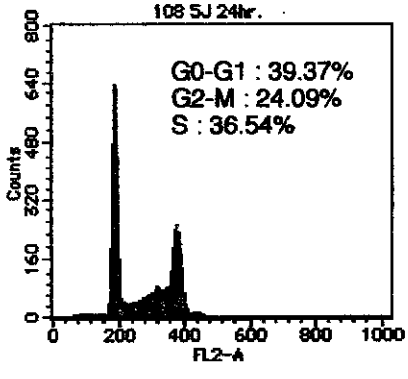


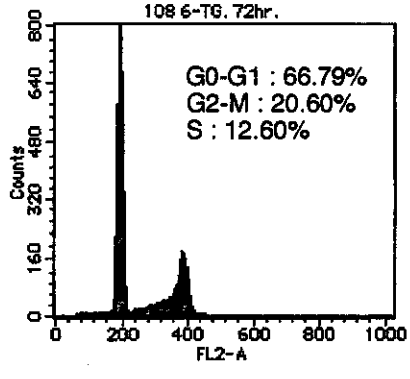




108 : XPA-/- cancer cell line







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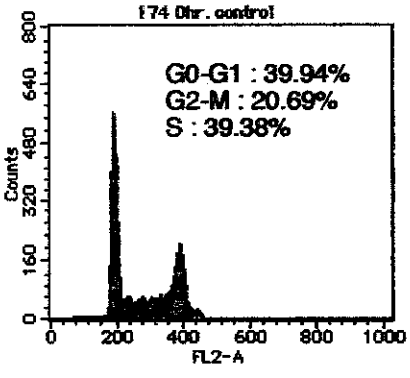

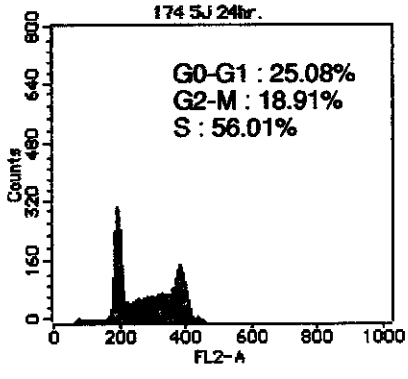

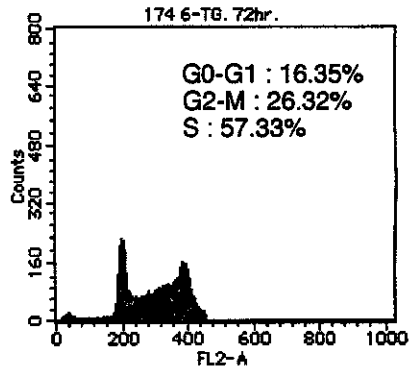

Protective Effects of Sunscreening Agents on Photocarcinogenesis, Photoaging, UV Immunosuppression, and DNA Damage in XPA gene Knock Out Mice

Satoshi Horiki*, Hiroko Miyauchi-Hashimoto*, Kiyoji Tanaka †,
Osamu Nikaido ¶, Takeshi Horio*

*Department of Dermatology, Kansai Medical University, Osaka, Japan

† Institute for Molecular and Cellular Biology, Osaka University, Suita, Osaka, Japan

¶ Kanazawa University Division of Radiation Biology, Kanazawa, Japan

Correspondence and reprint requests: Takeshi HORIO,
Department of Dermatology, Kansai Medical University,
10-15, Fumizono-cho, Moriguchi, Osaka, 570-8506, Japan
Tel:+81-6-6692-1001 Fax:+81-6-6996-3299

ABSTRACT

We investigated the protective effects of commercial sunscreening agents against UVB-induced photoresponses in group A xeroderma pigmentosum (XPA) model mice. SPF 10 and SPF 60-sunscreens protected partially and almost completely, respectively, ear swelling responses produced by UVB up to 200 mJ/cm^2 in (-/-) mice. XPA (-/-) mice were irradiated at the cumulative doses of 2.6 J/cm^2-UVB during the period of 24 wks with or without SPF 10 or SPF 60 sunscreen. UV-induced skin tumors developed in all unprotected (-/-) mice (13.3 tumors/mouse). SPF 60 sunscreen afforded stronger protection against photocarcinogenesis (1.0 tumors/mouse) than SPF 10 sunscreen (4.4 tumors/mouse). Regarding photoaging, SPF 60 sunscreen also protected against mast cell infiltration (79% inhibition) and elastin accumulation in XPA (-/-) mice compared with unprotected (-/-) mice. In XP (-/-) mice, local and systemic immunosuppression was induced at 20 mJ/cm^2 of acute UVB irradiation by 39.4 % and 33.2 % as compared with unirradiated positive control mice, respectively. SPF 60 sunscreen partially protected against local immunosuppression, but 22.4 % suppression was still observed in SPF 60-treated and 20 mJ/cm^2-UVB irradiated (-/-) mice. SPF 10 sunscreen did not protect against local immunosupression. Moreover, systemic immunosuppression was not significantly protected with both sunscreening agents. Formation of cyclobutane pyrimidine dimer (CPD) was estimated by immunofluorescent staining using monoclonal antibody. In (-/-) mice, SPF60 sunscreen provided stronger protection against CPD formation after 200 mJ/cm^2-UVB irradiation than SPF 10 sunscreen. Our results suggest that SPF 60 or higher sunscreening agents should be used to protect against increased photoresponses in XP patients.

INTRODUCTION

Xeroderma pigmentosum (XP) is a genetically heterogenous group of autosomal ressesive diseases characterized by defective excision repair of damaged DNA (Robbins, 1974). Excessive photosensitivity, accelerated photoaging, and a high incidence of skin tumors at an early age are commonly observed. In Japan, group A XP (XPA) is the most common form of this disease, and exhibits the severest clinical symptoms including UV-induced skin cancer among all complementation groups.

UV irradiation induces dipyrimidine lesion in DNA including the formation of cyclobutane pyrimidine dimers (CPD) (Hart, 1977), pyrimidine (6-4) pyrimidone photoproducts, and other minor lesions (Franklin, 1985). It is widely accepted that UV-induced DNA damage and repair mechanisms are highly relevant to UV-induced skin cancers. Immunosuppression can also enhance the incidence of UV-induced skin tumors in mice models (Kripke, 1976) (Fisher, 1982) and in human (Streilein, 1994). Because the UV-induced tumors in mice are highly antigenic (Kripke, 1974), these tumors do not grow progressively but are rejected when transplanted in normal syngeneic recipent mice. However, the transplanted tumors can be accepted in the mice which have been previously exposed to UVB. It was demonstrated that exposure to subcarcinogenic doses of UVB suppressed the generation of cell-mediated immune reactions by inducing the production of antigen-specific suppressor T cells. We have previously indicated that UVB irradiation greatly enhanced the immunosuppression and also inhibited natural killer cell activity in XPA (-/-) mice (Miyauchi-Hashimoto, 1996, 1999). Thus, the development of UV-induced skin cancer in XP patients may be enhanced by UV immunosuppression and a defective DNA excision repair mechanisms.

In patients with XP, photoaging, characterized by scaly, dry, wrinkled, and freckled skin is commonly observed at an early age (Cleaver, 1989). Of paticular important, photoaged skin includes populations of mutated cells which are not yet cancerous but have not been removed by the immunologic surveillance systems (Yaar,

1998). Therefore, protection against photoaging may also lead to suppression of photocarcinogenesis.

Commercial sunscreen lotion is generally accepted as being protective against sunburn by absorbing and/or reflectioning solar irradiation in daily life of healthy population. The Sun Protection Factor (SPF) value of commercial sunscreening agents is determined by the ability to inhibit the erythema induced by acute UV irradiation from a solar simulator which emits wavelength at 290-400 nm (FDA, 1993). Because the action spectrum inducing pyrimidine dimer formation in the DNA of human skin *in vivo* resembles the erythema action spectrum (Sutherland, 1981), high SPF sunscreen is often advocated as a means for preventing the cumulative effect of solar exposure. In fact, high SPF sunscreen significantly suppresses photocarcinogenesis in mouse model (Kligman, 1980), and in human (Naylor, 1995). Likewise, SPF 15 sunscreen provides stronger protection against photoaging than SPF < 15 sunscreen (Uitto, 1998). However, photoaging and UV skin tumors are not induced by acute UV irradiation, but by chronic UV exposure. Moreover, the SPF value does not necessarily correlate with protection against immunosuppression and DNA damage that can occur even with doses of UV exposure that do not cause erythema (Homey, 1997). Therefore, it is still a open question whether high SPF sunscreen can offer higher protection against UV carcinogenesis or photoaging. Nevertheless, higher SPF sunscreens are prone to be recommended for patients with XP than for healthy population. They should be educated along the photoprotection strategies based on the evidence obtained from *in vivo* experiments. In this regard, animal model has been required.

Recently, Nakane *et al* have developed an XPA gene-deficient mouse (XPA mouse) by a gene-targeting technique (Nakane, 1995). This mouse is defective in the nucleotide excision repair mechanism and has a high incidence of UV-induced skin tumors. Furthemore, our laboratory has previously described that this XPA mouse has greatly enhanced UVB-induced local and systemic immunosuppression and can serve as a model

for human XPA (Miyauchi-Hashimoto, 1996). A further aim of this study was to estimate the protective effects of sunscreening products against the increased photosensitivity in XPA mice.

Materials and Methods

Animals

The details about XPA gene-deficient mice have been published elsewhere (Nakane, 1995). Their chimeric genetic backgrounds were CBA, C57BL/6, ICR, and HR-1.6. These mice were bred in the animal center of Kansai Medical University Animal Centre in specific pathogen-free conditions. For *in vivo* experiments, at least five animals were used in each group at 7 to 10 wk of age. The mice were anesthetized by intraperitoneal injection of pentobarbital to keep them immobile during experiments. Since the mice are hairless, hair removing is not needed. Our experimental protocols are along the Rules of the Animal Experimentation Committee, Kansai Medical University.

UV Irradiation

The UVB source was a bank of seven fluorescent sunlamps (FL.20SE.30; Toshiba Medical Supply, Tokyo, Japan) with an emission spectrum of 275 to 375 nm, peaking at 305nm. The irradiance of UVB was measured by a radiometer (UVR-305/365D(Ⅱ); Toshiba Medical Supply).

Sunscreen agents

Two sunscreen products (Shiseido, Tokyo, Japan) with different sun protection factors (SPF 60 and SPF 10) were evaluated. These sunscreens or their vehicles were applied evenly to the back skin at 0.2 mg/cm^2 at least 20 min before the UVB irradiation. SPF 60 sunscreen agent (ANNESSA P Sunscreen) contains W/O cream including octyl methoxycinnamate, titanium dioxide, and zinc oxide. SPF10 suncreen contains W/O cream including 7% octyl methoxycinnamate.

UV inflammation

The ears of (+/+) and (-/-) mice were irradiated at 5, 20, 100, or 200 mJ/cm^2 of UVB. Ear

thickness was measured with dial thickness gauge (Peacock, Tokyo, Japan) immediately before and after UV irradiation for 5 consecutive days. Sunscreen agent was applied evenly to both aspects of the ear at a dose of 0.2 mg/cm^2. Ear swelling response was measured as the increment in thickness above the baseline value.

Induction of UV tumor

At first, we compared the protective effect of SPF 60 sunscreen on UV carcinogenesis. XP (+/+) and (-/-) mice with or without SPF 60 sunscreen were irradiated on the back three times a week at a dose of 20 mJ/cm^2. At least twenty mice were used in each group. The cumulative irradiation dose was 1 J/cm^2 over 17 wk (50 irradiations). Second, we compared SPF 60 with SPF 10 sunscreen. XP (-/-) mice with or without sunscreen application were irradiated with UVB starting at 20 mJ/cm^2. The UV dose was increased by 5 mJ/cm^2 at 2 wk intervals up to 40 mJ/cm^2. The cumulative dose was 2.6 J/cm^2 over 24 wk (71 irradiations).

Tumor counting

The number of tumors was counted on the dorsal area, where test materials were applied, excluding the head and ears. The appearance and development of skin tumors were checked weekly by counting the number and classifiying by diameter; the number of all tumors, tumors larger than 3 mm, and tumors larger than 5 mm in diameter.

Histological examination

Strips of UV-irradiated dorsal skin were removed at 4 wk after completion of UVB irradiation at cumulative dose of 2.6 J/cm^2 (71 irradiations). Skin sections were fixed in 10% formaldehyde and embedded in paraffin and stained routinely with hematoxylin and eosin (H&E), occasionally with truidin-blue for mast cells, and Luna's aldehyde fuchsin for elastic fibers (Kligman, 1981). To assess photoaging, stained mast cells were counted in five randomly selected fields per section with the aid of a calibrated ocular grid.

Induction of Immunosuppression

In order to induce immunosuppression, mice were exposed to UVB at a dose of 20

mJ/cm^2 on the abdomen. Twenty-four h later, mice were contact sensitized by painting 25 μl of 1% DNFB in acetone:olive oil (4:1) on the exposed abdomen or on the non-exposed back to induce local or systemic immunosupppression, respectively. In groups of mice, sunscreening agents or vehicle had been applied before UVB exposure. During irradiation, ears of all mice were shielded. For elicitation of CHS, both aspects of the ears were painted with 20 μl of 0.2% DNFB 7 d after the sensitization. Twenty-four h later, CHS was estimated by the ear swelling response. Ear thickness was measured with a dial thickness gauge (Peacock, Tokyo, Japan) before and 24 h after the challenge dose, and the difference between the two readings was recorded as the ear swelling.

Immunofluorescent staining for CPD

Cyclobutane pyrimidine dimers were detected by immunofluorescence staining using mouse monoclonal antibodies. XP (+/+) and (-/-) mice were UVB-irradiated at a dose of 50, 200, and 500 mJ/cm^2 on the back with or without sunscreen agent. Immunofluorescence of CPD staining was followed at 1, 3, 6, 12, 24, 48, and 72 h after UVB irradiation. The staining procedure for detection of CPD has been previously described in detail (Muramatu, 1992). In breif, skin sections (5 μm) were prepared using a cryostat. These sections were air dried and DNA was denatured by 0.07 N NaOH in 70% ethanol for 4 min. Slides were incubated at room temperature for 30 min in PBS containing 1% newborn calf serum (Nacalai Tesque, Kyoto, Japan). The sections were stained with CPD monoclonal anti-mouse IgG (diluted at 1:50 in PBS) antibody and, thereafter, stained with goat-mouse IgG Fluorescein isothiocyanate (FITC)(Becton Dikinson) (diluted at 1:50 in PBS) for 1h. The slides were mounted in buffered gycerol (Lipshaw, Immunon. Pittsburgh, USA). Confocal microscopy (Fluoview, OLYMPUS) was performed with 488 nm excitation lines from a krypton/argon laser. Excitation and collected emission (FITC, 535 nm band passfilter) were performed simultaneously for both labels. The intensity of immunofluorescence was graded by its fluorescent intensity.

Statistical evaluation

Each value represents the mean ± SD. Statistical significance was assessed by Student's t-test.

Results

UV Inflammation

In (+/+) mice, UVB irradiation at a dose of greater than 200 mJ/cm^2 caused significant ear swelling response, peaking at day 3 (**Figure 1 A**). The SPF 60 sunscreen provided more protection than SPF 10 sunscreen (**Figure 1 B**). In (-/-) mice, UVB irradiation, at a dosage greater than 20 mJ/cm^2, caused greater and longer-lasting ear swelling response than with 200 mJ/cm^2 in (+/+) mice. SPF 60 sunscreen was more effective in suppressing UV inflammation in XPA (-/-) mice than SPF 10 sunscreen (**Figure 1 C, D, E, F**).

Tumor production

UV-induced tumors were observed only in (-/-) mice exposed to UVB at the dose examined. **Figure 2 A** shows the time course of skin-tumor formation in (-/-) mice exposed to UVB irradiation with and without SPF 60 sunscreen. In unprotected (-/-) mice, tumors first occurred 9 wk after initiation of UVB exposure at total dose of 660 mJ/cm^2 (33 irradiations). At the completion of the UVB irradiation for 17 wk, at a total dose of 1 J/cm^2 (50 irradiations), tumors were observed in all unprotectetd (-/-) mice (7 tumors/mouse). On the other hand, few tumors were found in the SPF 60 sunscreen-protected (-/-) mice (0.08 tumors/mouse). Tumor formation and growth continued even after completion of irradiation, developing 25 tumors/mouse at 21 wk in unprotected (-/-) mice.

Figure 2 B illustrates the tumor formation in (-/-) mice exposed to UVB at a total dose of 2.6 J/cm^2 with or without application of SPF 60 or SPF 10-sunscreen. In non-UV-irradiated group of (-/-) mice, no tumor was observed. In UV-irradiated, unprotected group, tumors first appeared at 10 wks after a cumulative dose of 860 mJ/cm^2 (27 irradiations). In contrast, in SPF10- or SPF-60-treated mice, tumors developed later,

appearing at 16 wk (1.72 J/cm^2, 49 irradiations) or 17 wk (1.84 J/cm^2, 52 irradiations), respectively. At the completion of the UV irradiation, at a cumulative dose of 2.6 J/cm^2 (71 irradiations), all mice in the unprotected group beared tumors (13.3 tumors/mouse). While SPF 60 sunscreen almost protected against UV-induced skin tumors (1.0 tumors/mouse), small skin tumors were observed in SPF 10-protected mice (4.375 tumors/mouse). There was no difference in tumor formation between non protected and vehicle-treated mice (data not shown). Mice bearing large tumors died during the observation period. Significant differences in UV-induced skin tumors between the SPF 60 and SPF 10 protected groups were detected in 24th wk at the final cumulative dose of 2.6 J/cm^2.

In regard to tumor development, unprotected (-/-) and SPF 10-protected mice beard tumors of >5 mm in diameter at the completion of UVB irradiation (2.0 and 0.13 tumors/mouse, respectively) (**Table 1**). Likewise, unprotected (-/-) and SPF 10-protected mice beard tumors of >3 mm in diameter (4.33 and 1.13 tumors/mouse, respectively). In contrast, SPF 60-protected mice beared no tumor of >3 mm in diameter was found at the completion of UVB irradiation.

Figure 3 shows the mice at 6 wk after the completion of UVB irradiation. In unprotected, UV-irradiated (-/-) mice, multiple large skin tumors were found, which were SCCs of varying malignancy on histology (data not shown). In addition, dry skin and scaling, which are consistent with the clinical findings of XP patients, are prominent. In SPF 60 sunscreen-protected (-/-) mice, few tumors were found in the protected area. On the other hand, SPF 10-protected (-/-) mice beared several small squamous cell carcinomas. Also, signs of photoaging, such as skin thickening and wrinkling, were apparent in SPF 10-protected (-/-) mice. No tumors were found in non-UVB irradiated mice. In both experiments, no severe sunburn reaction was observed throughout the course of irradiation.

Photoaging

Figure 4 shows Luna's stain of UV-exposed (2.6 J/cm^2, 71 irradiations) dorsal skin from a (-/-) mouse with and without sunscreen. While only few elastin was detected in the non-UV-irradiated group, pronounced elastin accumulation and epidermal hyperplasia were seen in UV-irradiated unprotected (-/-) mice. SPF 60 sunscreen almost fully protected against elastin accumulation and epidermal hyperplasia, whereas significant elastin accumulation and epidermal hyperplasia were seen in SPF 10-protected (-/-) mice. Vehicle application did not protect against accumulation of elastin and epidermal hyperplasia at all (data not shown).

As shown in **Table 2**, the number of dermal mast cell was significantly greater in unprotected (-/-) mice than non UVB-irradiated control mice. SPF 60 sunscreen protected against dermal mast cell accumulation by 79 %, while SPF 10 sunscreen did not protect, but rather enhanced by 44 %. Vehicle treatment did not protected against dermal mast cell accumulation at all (data not shown). **Figure 5** shows the truidine-blue stain for mast cells. In unprotected UVB-irradiated (-/-) mice, excessive accumulation of mast cells was observed. Of note, a dissection of collagen by mast cell infiltration and its granulation which is compatible with enzymic digestion pattern was prominent. No remarkable mast cell infiltration was observed in SPF 60-protected (-/-) mice. On the other hand, mast cell infiltration with degranulation is prominent in SPF 10-protected mice. In non-UVB-irradiated mice, mast cells were found in small numbers. Vehicle application protect against neither mast cell accumulation nor degranulation (data not shown).

Local immunosuppresion of CHS

In (+/+) mice at 20 mJ/cm^2 of UVB irradiation, local immunosuppression was noted (**Figure 6 A**). In unprotected UV-irradiated mice, CHS was suppressed by 29.8 %. In SPF 60- and SPF 10- protected mice, CHS was suppressed by 12.4 % and 28.9 % respectively. On the other hand, in (-/-) mice that were not protected showed an 39.4 % reduction in CHS response compared with non UV-irradiated contact sensitized mice.

Mice protected with SPF 60 and SPF 10 showed a 22.4 % and 34.5 % reduction in CH response, respectively. Sunscreening materials did not influence CHS by themselves (data not shown).

Systemic immunosuppresion of CHS

In (+/+) mice exposed to 20 mJ/cm^2 of UVB irradiation, no significant systemic immunosuppression was detected (**Figure 6 B**). In unprotected UV-irradiated (-/-) mice, CHS was suppressed by 33.2 % compared with non UV-irradiated mice. Sunscreening agents with SPF 10 and 60 did not protect against the UVB-induced systemic immunosuppression.

CPD formation

Unirradiated (+/+) and (-/-) mice skin showed no evidence of CPD immunoreactivity. Dose-dependent induction of nucelar immunofluorescent staining of CPD was detected in (+/+) and (-/-) mice (data not shown). No difference of fluorointensity between (+/+) and (-/-) mice was detected at 200 mJ/cm^2 and 500 mJ/cm^2-UVB exposures. The intensity of nuclear immunofluorescence was greater in the lower than in the upper epidermal layers (**Figure 7**).

In (+/+) mice, at 50 mJ/cm^2 of UVB irradiation, nuclear immunofluorescence of CPD was observed at 1 h after UVB irradiation. Thereafter, it was gradually decreased and almost disappered 24 h after UVB irradiation. However, at 500 mJ/cm^2 of UVB irradiation, immunofluorescent staining of CPD was long-lasting, which is still detectable even 72 hrafter UVB irradiation in (+/+) mice. In (-/-) mice, at 50 mJ/cm^2 of UVB irradiation, nuclear immunofluorescence of CPD was observed at 1 h after UVB irradiation, and lasted even 72 h after UVB irradiation.

We compared the ability of sunscreen to protect against CPD immunofluorescent staining at a dose of 200 mJ/cm^2 12 h after UVB irradiation in (-/-) mice (**Figure 8**). In (-/-) mice, it was almost undetectable in SPF 60-protected mice, whereas, in SPF 10-protected mice, it was detected with less intense than that of unprotected (-/-) mice.

DISCUSSION

A number of investigations of cellular and molecular biology are resolving pathomechanims of UVR-induced skin cancers in patients with XP. At the present time, however, only photoprotection can prevent increased photoresponses in XP. Characteristic, clinical features of XP patient skin include severe and long-lasting sunburn, enhanced photoaging, and early and easy development of skin tumors, which are most prominent in complementation group A-XP. These cutaneous manifestations can be reproduced in XPA gene knock out mice (Miyauchi-Hashimoto, 1999). Furthermore, we have demonstrated that local and systemic immunosuppression were enhanced and natural killer cell activity was greatly impaired after UVB exposure in the model mice (Miyauchi-Hashimoto, 1999). An animal model is a relevant strategy to clinical investigations of human disease. In the present study, we evaluated protective effects of commercially available sunscreening agents on increased photoresponses in XPA (-/-) mice.

As expected, high SPF sunscreen agent strongly suppressed UV-inflammation in XP (-/-) mice as well as in XP (+/+) mice. This implies that sunscreen agent may also reduce enhanced sunburn reaction in patients with XP. Furthermore, it is conceivable that sunscreen agent suppresses not only UV-inflammation, but also photocarcinogenesis, because the action spectrum inducing DNA damage resembles the erythema action spectrum (Sutherland, 1981). Although sunscreening products were essentially developed to protect against acute sunburn, experimental studies have tumor formation (Gasparro, shown that ??
1998) can be also protected by some sunscreens. There are also clinical observations which suggest that the use of sunscreens can reduce the risk of non-melanoma skin cancers in healthy (Flindt-Hansen, 1980) (Kligman, 1980) (Wulf, 1982) and XP humans (Kondoh, 1994). The protective efficiency of sunscreens is estimated based on their ability to suppress UV-induced erythema in human skin as expressed by SPF value.

The data presented in this study clearly demonstrated that SPF 60 sunscreen more