Photomutagenicity of Retinyl Palmitate by Ultraviolet A Irradiation in Mouse Lymphoma Cells

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Abstract

Retinyl palmitate (RP), a storage form of vitamin A, is frequently used as a cosmetic ingredient, with more than 700 RP-containing cosmetic products on the U.S. market in 2004. There are concerns for the possible genotoxicity and carcinogenicity of RP when it is exposed to sunlight. To evaluate the photomutagenicity of RP in cells when exposed to ultraviolet A (UVA) light, L5178Y/$Tk^{+/-}$ mouse lymphoma cells were treated with different doses of RP alone/or in the presence of UVA light. Treatment of the cells with RP alone at the dose range of 25–100 μg/ml did not increase mutant frequencies (MFs) over the negative control, whereas treatment of cells with 1–25 μg/ml RP under UVA light (82.8 mJ/cm^2/min for 30 min) produced a dose-dependent mutation induction. The mean induced MF (392×10^{-6}) for treatment with 25 mg/ml RP under UVA exposure was about threefold higher than that for UVA alone (122×10^{-6}), a synergistic effect. To elucidate the underlying mechanism of action, we examined the mutants for loss of heterozygosity (LOH) at four microsatellite loci spanning the entire chromosome 11, on which the *Tk* gene is located. The mutational spectrum for the RP + UVA treatment was significantly different from the negative control, but not significantly different from UVA exposure alone. Ninety four percent of the mutants from RP + UVA treatment lost the Tk^+ allele, and 91% of the deleted sequences extended more than 6 cM in chromosome length, indicating clastogenic events affecting a large segment of the chromosome. These results suggest that RP is photomutagenic in combination with UVA exposure in mouse lymphoma cells, with a clastogenic mode-of-action.

Keywords

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INTRODUCTION

Vitamin A, a natural retinoid, is required for many biological processes including cell growth, differentiation, and maintenance (Tee, 1992). Retinyl palmitate (all-*trans*-retinyl palmitate, RP) is a principal storage form of retinol in humans and animals, and it can be enzymatically hydrolyzed back to retinol *in vivo* (Idson, 1990). Because retinol is thermally unstable and RP is relatively more stable, RP is widely used as a special interest ingredient in cosmetic formulations and was present in more than 700 products available on the U.S. market in 2004 (FDA, 2004). The Cosmetic Ingredient Review Expert Panel, the cosmetic industry's group for evaluating the safety of cosmetic ingredients, concluded in 1987 that RP is safe as a cosmetic ingredient in the present practices of use and in concentrations up to approximately 10% (Cosmetic Ingredient Review, 1987). These products include skin care and moisturizing preparations, suntan lotions, cosmetic makeup, and lipsticks. The topical application of RP to the skin of humans can produce significant levels of RP both inside and on top of the skin, where it can interact with sunlight (Ihara *et al.*, 1999). The possible toxic effects of RP on skin exposed to sunlight have not been fully investigated. Therefore, RP was referred by the U.S. Food and Drug Administration (FDA) to the National Toxicology Program (NTP) as a high priority compound for phototoxicity and photocarcinogenicity studies, primarily based on its increasingly widespread use on sun-exposed skin and the association between topical application of retinoids and enhancement of photocarcinogenesis (Fu *et al.*, 2002).

The UV radiation that reaches the surface of the earth from the sun is divided into two wavebands, UVA (between 320 and 400 nm) and UVB (between 290 and 320 nm), with visible and infrared light at longer wavelengths (FDA, 1999). Retinyl palmitate shows maximum UV-visible absorption at 326 nm (Tee, 1992). Therefore, UVA may play an important role in the photobiological activity of RP. UVA itself is a carcinogen and mutagen. UVA can induce cutaneous squamous cell carcinoma in mice (de Gruijl *et al.*, 1993; Matsui and DeLeo, 1991) and increase melanoma risk in humans (Swerdlow *et al.*, 1988; Walter *et al.*, 1990; Westerdahl *et al.*, 2000). UVA radiation increases mutant frequency (MF) and micronucleus formation (Phillipson *et al.*, 2002).

In our previous study on the effects of UVA on RP (Cherng *et al.*, 2005), we determined that photoirradiation of RP and its photodecomposition products generated reactive oxygen species (ROS) and resulted in lipid peroxidation. Generally, ROS and lipid peroxidation produce oxidative damage to DNA and result in mutations. Retinyl palmitate and its photodecomposition products in combination with UVA exposure, however, were not mutagenic in *Salmonella typhimurium* tester strains TA98, TA100, TA102, and TA104 in the presence or absence of S9 activation enzymes and were not photomutagenic in *S. typhimurium* TA102 when irradiated with UVA. Oxidative damage frequently results in mutations with large chromosomal alterations (Harrington-Brock *et al.*, 2003; Rothfuss *et al.*, 2000; Singh *et al.*, 2005; Takeuchi *et al.*, 1997), which cannot be detected by the *S. typhimurium* gene mutation test system. Therefore, we hypothesized that mutations generated by the UVA irradiation of RP may be due primarily to large-scale chromosome damage.

To test our hypothesis, we have used the L5178Y mouse lymphoma assay (MLA) to evaluate whether RP can interact with UVA to produce a photomutagenic effect. Unlike the microbial assays, the MLA detects a broad spectrum of genetic damage, including both point mutations and chromosomal mutations. This feature makes the MLA particularly useful for detecting mutational events that result from oxidative DNA damage. We also examined the types of mutations induced by UVA and RP + UVA to understand the underlying mechanism of action for the photomutagenicity of RP in combination with UVA exposure.

MATERIALS AND METHODS

Cells and culture conditions

The L5178Y/$Tk^{+/-}$ –3.7.2C mouse lymphoma cell line was used for the mutation assay. Cells were grown according to the methods described by Chen and Moore (2004). Briefly, the basic medium was Fischer's medium for leukemic cells of mice with L-glutamine (Quality Biological Inc., Gaithersburg, MD) supplemented with pluronic F68 (0.1%), sodium pyruvate (1 mM), penicillin (100 U/ml), and streptomycin (100 μg/ml). The treatment medium (F_{5p}), growth medium (F_{10p}), and cloning medium (F_{20p}) were the basic medium supplemented with 5%, 10%, and 20% heat-inactivated horse serum, respectively. The cultures were maintained in a humidified incubator with 5% CO_2 in air at 37°C. Unless otherwise noted, all culture supplies were purchased from Invitrogen Life Technologies (Carlsbad, CA).

Cell treatment with RP alone

Retinyl palmitate was purchased from the Sigma (St. Louis, MO). The RP working solution (100×) was prepared just prior to use by dissolving it with anhydrous dimethyl sulfoxide (DMSO). The cells were suspended in 100-mm-diameter tissue culture dishes at a concentration of 6×10^6 cells in 10 ml of treatment medium. Because we focused on the photomutagenicity of RP by UVA, we did not test the mutagenic potential of RP more than 100 μg/ml. One hundred μl of the RP stock solutions at concentrations between 25 and 100 μg/ml were added to the medium, and the cells were incubated for 4 h at 37°C. In all cases, including negative controls (DMSO only) and positive controls [0.1 μg/ml 4-nitroquinoline-1-oxide (4-NQO)], the final concentration of DMSO in the medium was 1%. After treatment, the cells were centrifuged and washed once with fresh medium and then resuspended in growth medium at a density of 3×10^5 cells/ml in 25-cm² cell culture flasks to begin phenotypic expression.

Cell treatment with UVA alone

The light box, a custom-made 4-lamp unit uses UVA lamps (National Biologics, Twinsburg, OH) (Cherng *et al.*, 2005). The irradiance of light was determined with an Optronics OL754 Spectroradiometer (Optronics Laboratories, Orlando, FL), and the light dose was routinely measured with a Solar Light PMA-2110 UVA detector (Solar Light Inc., Philadelphia, PA). The maximum emission of the UVA was between 340 and 355 nm. The light intensities at wavelengths below 320 nm (UVB light) and above 400 nm (visible light) were about two orders of magnitude lower than the maximum at 340–355 nm. The 6×10^6 cells in 10 ml of

treatment medium in 100-mm diameter tissue culture dish were exposed to UVA light at a rate of 82.8 mJ/cm^2/min for various times from 5 to 45 min.

Cell treatment with pre-irradiated RP

The pre-irradiated RP was obtained by irradiating the stock RP solution with 82.8 mJ/cm^2/min UVA for 30 min immediately before adding the solution to the cell culture to make a final concentration of pre-irradiated RP at 25 μg/ml. The cells were treated with the UVA pre-irradiated RP for 4 h at 37°C.

Cell treatment with RP and UVA.

Cells were treated with different concentrations (1–25 μg/ml) of RP and concomitantly exposed to 82.8 mJ/cm^2/min UVA for 30 min. The treated cultures were then incubated with the RP at 37°C for an additional 3.5 h.

The Tk microwell mutation assay

Mutant selection was performed as described previously (Chen and Moore, 2004). Briefly, the cells were counted and the densities were adjusted using fresh medium at approximately 1 and 2 days after exposure. For mutant enumeration, trifluorothymidine (TFT, 3 μg/ml) was added to the cell culture in cloning medium, and cells were seeded into four 96-well flat-bottom microtiter plates using 200 μl per well and a density of 2000 cells/well. For the determination of plating efficiency, approximately 1.6 cells were aliquoted in 200 μl per well into two 96-well flat-bottom microtiter plates. All plates were incubated at 37°C in a humidified incubator with 5% CO$_2$ in air. After 11 days of incubation, colonies were counted and mutant colonies were categorized as small or large. Small colonies are defined as those smaller than 25% of the diameter of the well. Mutant frequencies were calculated using the Poisson distribution. The induced MF was obtained by subtracting the background MF (negative control MF) from the MF in the treatment group. Cytotoxicity was measured using relative total growth (RTG), which includes a measure of growth during treatment, expression, and cloning (Chen and Moore, 2004).

Detection of loss of heterozygosity (LOH) at the Thymidine kinase (Tk1) and other three microsatellite loci spanning the entire chromosome 11 for Tk mutants

Mutant cells were directly taken from TFT-selected plates. Only mutants obtained from the 30-min UVA exposure, the concomitant treatment of 25 μg/ml RP and UVA, and the negative control were isolated and analyzed. The mutants from the RP treatment alone were not analyzed for LOH because RP was not mutagenic under the conditions used (see *Results*). The mutant cells were washed once with PBS by centrifugation, and cell pellets were quickly frozen and stored at −80°C. Genomic DNA was extracted by digesting the cells in lysis buffer (10 mM Tris-HCl [pH 7.5], 5 mM MgCl$_2$, 1% [v/v] Triton X-100, 1% [v/v] Tween 20) with 200 μg/ml of proteinase K at 60°C for 90 min, followed by inactivation of proteinase K at 95°C for 10 min. The procedure for the polymerase chain reaction (PCR) analysis of LOH at the *Tk* locus was performed as previously described (Chen *et al.*, 2002a). For PCR analysis of LOH at other loci (D11Mit42, D11Mit29, and D11Mit74 loci; Fig. 1), the amplification reactions were carried out in a total volume of 20 μl using 2×PCR master

Mix (Promega, Madison, WI) and pairs of primers described previously (Singh *et al.*, 2005). The thermal cycling conditions were as follows: initial incubation at 94°C for 3 min, 40 cycles of 94°C denaturation for 30 s, 55°C annealing for 30 s, and 72°C extension for 30 s, and a final extension at 72°C for 7 min. The amplification products were scored for the presence of one band (indicating LOH) or two bands (retention of heterozygosity at the given locus) after 2% agarose gel electrophoresis.

Statistical analyses

To evaluate the differences between the treatment groups, one-way analysis of variance (ANOVA) followed by the Student-Newman-Keul test using SigmaStat (SPSS Science, Chicago, IL) was used. Loss of heterozygosity patterns of mutants were compared using the computer program written by Cariello *et al.* (1994) for the Monte Carlo analysis developed by Adams and Skopek (1987).

RESULTS

The relative total growth (RTG) values, *Tk* MF, small colony MF, and large colony MF from one representative MLA experiment with treatment of RP, UVA, and RP + UVA are presented in Table 1. These results were replicated in two additional experiments and the data are displayed as mean ± 1 standard deviation in Figures 2 and 3.

A dose-related cytotoxicity and mutagenicity of UVA was observed after the L5178Y/$Tk^{+/-}$ mouse lymphoma cells were exposed to different doses of UVA (Fig. 2). The MFs increased linearly with UVA exposure time. Treatment of cells with RP alone (25, 50, and 100 μg/ml) for 4 h showed little cytotoxicity (the RTG at the highest dose was about 80%) and did not increase the MF (Fig. 3). Treatment of cells with various concentrations of RP concomitantly exposed to 82.8 mJ/cm^2/min for 30 min (2.48 J/cm^2) UVA, however, resulted in linear dose-dependent increases for both the cytotoxicity and mutagenicity (Fig. 3). Comparing all concomitant treatment groups (UVA + RP) with the UVA alone exposure, the 5, 10, and 25 μg/ml of RP under UVA exposure were significantly different from the UVA alone ($p < 0.05$, 0.01, and 0.001, respectively). The MFs for the dose points higher than 25 μg/ml RP in the combination exposure were not determined because of the high cytotoxicity and low plating efficiency. The MFs for the negative control, UVA alone of 30 min, and UVA + RP (25 cg/ml) were $52 ± 3\ 3\ 10^{-6}$, $174 ± 24\ 3\ 10^{-6}$, and $444 ± 54 × 10^{-6}$, respectively. The induced MF, obtained by subtracting the negative control MF from the total MF in the treated culture, for the concomitant treatment of 25 μg/ml RP and 2.48 J/cm^2 UVA ($392\ 3\ 10^{-6}$) was about threefold higher than that for 2.48 J/cm^2 UVA exposure alone ($122\ 3\ 10^{-6}$).

To determine the mutagenicity of the photodecomposition products of RP created by UVA irradiation, we pre-irradiated RP with 2.48 J/cm^2 in the absence of cells. The pre-irradiated RP then was used to treat the cells at a concentration of 25 μg/ml. The MF for the pre-irradiated RP treatment was higher than that for 25 μg/ml RP treatment alone, but lower than that for 2.48 J/cm^2 UVA treatment alone, although there were no statistically significant differences. The MF for the pre-irradiated RP treatment was significantly less than the concomitant cellular exposure of RP and UVA (Fig. 4).

Loss of heterozygosity analysis of the mutants was conducted using four microsatellite loci spanning the entire chromosome 11 (Fig. 1) to determine the types of the mutations. DNA samples were isolated from 48 large and 48 small mutant colonies for the DMSO negative control, 2.48 J/cm^2 UVA treatment alone, and the concomitant treatment of 25 μg/ml RP and 2.48 J/cm^2 UVA, respectively. The percentages and MFs of different types of mutations for large and small colonies are displayed in Figure 5. Statistical analysis of the spectra revealed that the mutational spectra induced by UVA or RP + UVA were significantly different from the negative control, respectively, for both large and small colonies, whereas there was no statistical difference between the mutational spectra induced by UVA and RP + UVA treatments for both large and small colonies. A statistical pairwise multiple comparison of these mutational spectra is listed in Table 2. The most common type of mutation for UVA and RP + UVA was the LOH extending to *D11Mit42*, an alteration of DNA larger than 6 cM, whereas the major type of mutation in the control was non-LOH, indicating intragenic mutations in the *Tk* gene (Fig. 5).

DISCUSSION

The number of cosmetic retail products containing RP has increased rapidly in the last two decades because of the beneficial effects of RP on the appearance of skin. As a result, a growing number of products containing RP are being applied to sun-exposed skin. The biological consequences of photoactivation of RP by UVA light deserve investigation. Retinoids are effective chemopreventive agents against skin, head and neck, lung, breast, liver, and other forms of cancer (Hansen *et al.* 2000). However, retinoids administered orally are not effective in preventing skin papillomas and carcinomas caused by UV light (Hill and Grubbs, 1992). The effects of retinoids on experimental animal photocarcinogenesis have been reported (Kligman, 1987; Mikkelsen *et al.*, 1998). Supplementation of diets with vitamin A appeared to enhance UV carcinogenesis in hairless mice (Mikkelsen *et al.*, 1998). To explore the possible photomutagenicity of RP by UVA and its underlying mechanisms, we conducted studies to investigate the genotoxicity of RP in combination with UVA exposure in a previous study (Cherng *et al.*, 2005) and this study.

Retinyl palmitate alone, in concentrations of 25–100 μg/ml, did not increase the MF over control (Fig. 3), which is consistent with our previous study on RP mutagenicity using the *S. typhimurium* mutation test system (Cherng *et al.*, 2005). In the present study, the combined treatment of RP in concentrations of 1–25 μg/ml with a UVA dose of 2.48 J/cm^2 resulted in a significant increase of MFs, in a dose-dependent manner. The induced MF (subtracting the MF of the negative control from the MF observed in the test culture) at the 25 μg/ml RP with UVA exposure was about threefold higher than that for UVA-irradiation alone (Table 1). This is different from our previous studies using the microbial mutation assay (Cherng *et al.*, 2005) in which RP treatment of *S. typhimurium* TA102 under UVA irradiation was not mutagenic. This inconsistency is most likely related to the fact that combined treatment of RP and UVA causes clastogenicity. Compounds acting primarily by a clastogenic mechanism induce detectable mutagenicity in the MLA but are only weakly mutagenic or nonmutagenic in the microbial assays (Chen *et al.*, 2002b).

In this study, most of the mutants from concomitant treatment of RP and UVA were small colony mutants (66%, at a dose of 25 μg/ml, a MF of 287 of a total MF of 437), whereas the percentage of small colony mutants in the negative control cultures was 46% (a MF of 26 of a total MF of 57). Also, LOH at the *Tk* locus occurred in 85% in large colony mutants and 100% in small colony mutants (a total of 94% overall) from RP + UVA treatment, indicating that the photomutagenicity of RP by UVA irradiation results from a clastogenic mode of action. In the MLA, compounds that induce point mutations result in a high proportion of large colony *Tk* mutants and little LOH at the *Tk* locus, whereas clastogens tend to result in a high proportion of small colony mutants and predominantly LOH at the *Tk* locus (Applegate *et al.*, 1990; Chen *et al.*, 2002a, 2002b; Harrington-Brock *et al.*, 2003). LOH is an important mutational event in tumorigenesis and is frequently observed in a variety of human cancers at loci that are tumor-suppressor genes. LOH can result from any of several mechanisms, including large deletions, mitotic recombination, and whole chromosome loss (Honma *et al.*, 2001). Generally, depending on the severity of DNA damage of the *Tk* mutants, LOH will also occur at other loci along chromosome 11 in addition to the *Tk* gene.

To determine whether RP under UVA irradiation produces mutations through its photodecomposition products or through short-lived products like ROS and lipid peroxides, we irradiated RP for 30 min in the absence of cells, and the resulting pre-irradiated RP reaction mixture then was used to treat cells. The MF for pre-irradiated RP treatment was significantly lower than that for the concomitant exposure to RP and UVA, and it was not significantly different from that for RP treatment or the DMSO control (Fig. 4). This is consistent with our previous results that RP and its identified photodecomposition products did not bind with DNA in the presence of microsomal metabolizing enzymes (Cherng *et al.*, 2005). These results indirectly demonstrate that UVA irradiation of RP produces short-lived species like ROS that damage DNA and result in mutations.

Ultraviolet light causes DNA damage both directly and indirectly. Direct DNA damage is principally by UVB, forming cyclobutane pyrimidine dimers, pyrimidine-pyrimidones, and point mutations. Indirect DNA damage is principally caused by UVA-dependent photoactivation of organic compounds that generates short-lived species (Brendler-Schwaab *et al.*, 2004). Our previous research has shown that irradiation of RP with UVA light can generate ROS and lipid peroxides (Cherng *et al.*, 2005). Photodynamic action results in the production of free radicals, including ROS. Because of their high reactivity, ROS can attack all cellular constituents, including proteins, nucleic acids, and lipids. Hydroxyl radicals can initiate a chain reaction that produces multiple lipid hydroperoxide molecules from a single initial event (Gutteridge and Halliwell, 1990). The chain reaction is in effect amplifying the initial oxidative insult, and the resulting active oxygen species can damage DNA and produce mutations.

UVA itself induces genetic damage in cells via an oxidative stress mechanism (Drobetsky *et al.*, 1995; Kamiya, 2003; Phillipson *et al.*, 2002). In this study, the mutational spectra induced by UVA and RP + UVA are not significantly different ($p = 0.448$ in large colonies and $p = 0.816$ in small colonies; Table 2). This similarity suggests that RP + UVA induces mutations through the same mechanism as UVA, mainly oxidative DNA damage.

Chemicals that absorb UVA and visible light and generate ROS constitute the largest class of photosensitizers. After absorption of UVA light, RP potentially acts as a photosensitizer (Fu *et al.*, 2003). The mutagenicity of photosensitizers probably results from oxidative radicals formed in the photosensitization reaction (Rerko *et al.*, 1992). Several studies using different oxidative agents provide evidence that oxidative DNA damage generated from these agents can lead to different types of mutations, with a large proportion of chromosome mutations that result mainly from chromosome breakage (Harrington-Brock *et al.*, 2003; Nakajima *et al.*, 2002; Rothfuss *et al.*, 2000; Takeuchi *et al.*, 1997). In a recent study on the mutagenicity of the lipid peroxidation product 4-hydroxynonenal (4-HNE) in mouse lymphoma cells (Singh *et al.*, 2005), we found that the major type of mutation was LOH extending to *D11Mit42* (see Fig. 1 for the position), the same mutation as currently seen with RP + UVA treatment. The *Tk* mutants from cells exposed to either of these two compounds are about 60% this type of LOH mutation. A mechanistic pathway initiated by photoirradiation of RP with UVA light leading to induction of chromosome mutations has been suggested and is illustrated in Figure 6.

In summary, a combined treatment of RP and UVA irradiation produced a clearly synergistic photomutagenic effect at the heterozygous *Tk* locus in mouse lymphoma cells. Most of the *Tk* mutants induced by the concomitant treatment were the result of LOH, indicating a clastogenic mode of action. Oxidative DNA lesions are likely responsible for the photomutagenicity from the combined exposure of RP and UVA irradiation. These *in vitro* results suggest that the biological consequences of concomitant exposure to RP and UVA warrants further investigation.

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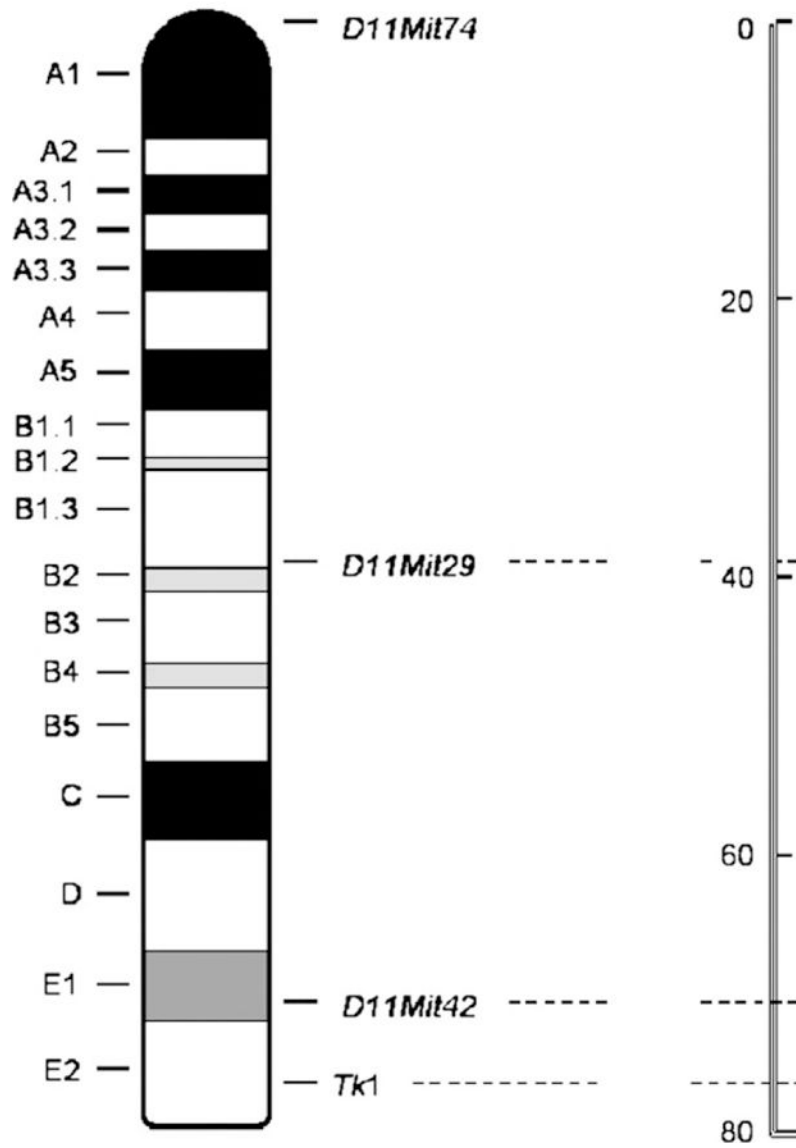
FIG. 1.
Ideogram of mouse chromosome 11. The loci that were analyzed for LOH (*Tk*1, *D11Mit74*, *D11Mit29*, and *D11Mit42*) are marked. The right ruler in cM indicates the distance from the top of the chromosome.
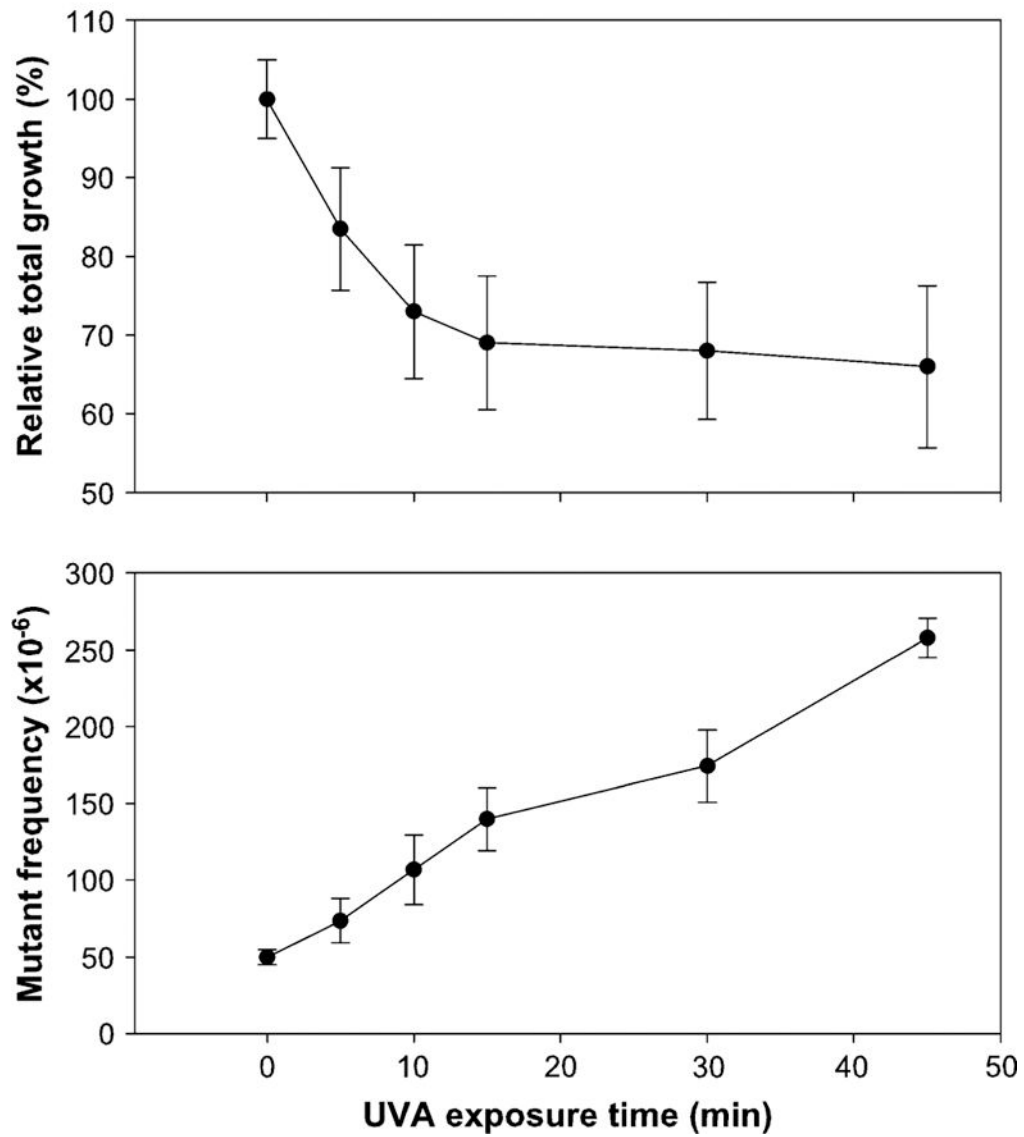
FIG. 2.
Cytotoxicity and mutagenicity of UVA in mouse lymphoma cells. The cells received 82.8 mJ/cm^2/min UVA irradiation for different periods of time. Cytotoxicity (defined as relative total growth) is shown in the top panel, and *Tk* mutant frequency is displayed in the bottom panel. The data points represent the mean ± 1 standard deviation from three independent experiments.
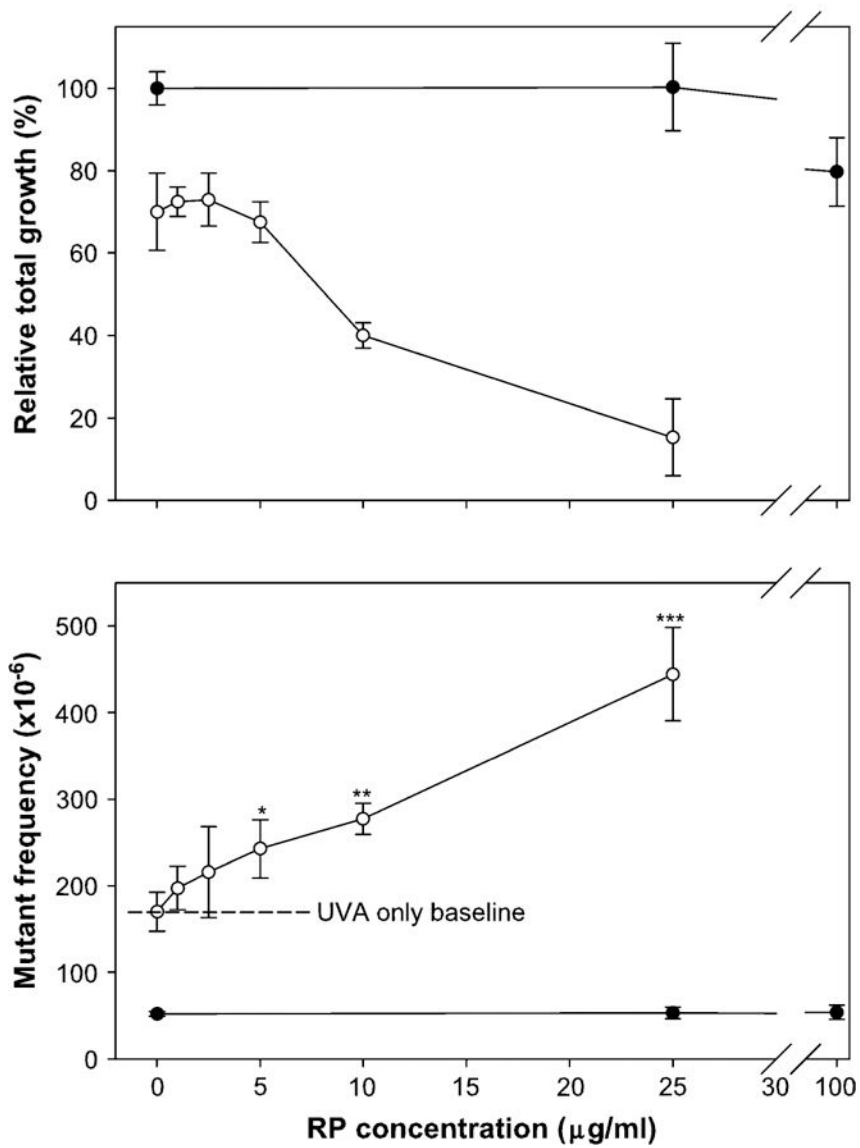
FIG. 3.

Comparison of cytotoxicity (the top panel) and mutagenicity (the bottom panel) of retinyl palmitate (RP) and RP + UVA in mouse lymphoma cells. The cells were treated with different concentrations of RP in conjunction with 82.8 mJ/cm^2/min UVA irradiation for 30 min (open circle) or without UVA irradiation (closed circle). The data points represent the mean ± 1 standard deviation from three independent experiments. The concomitant treatment groups (UVA + RP) with 5, 10, and 25 μg/ml of RP were significantly higher than the UVA alone (*$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$).
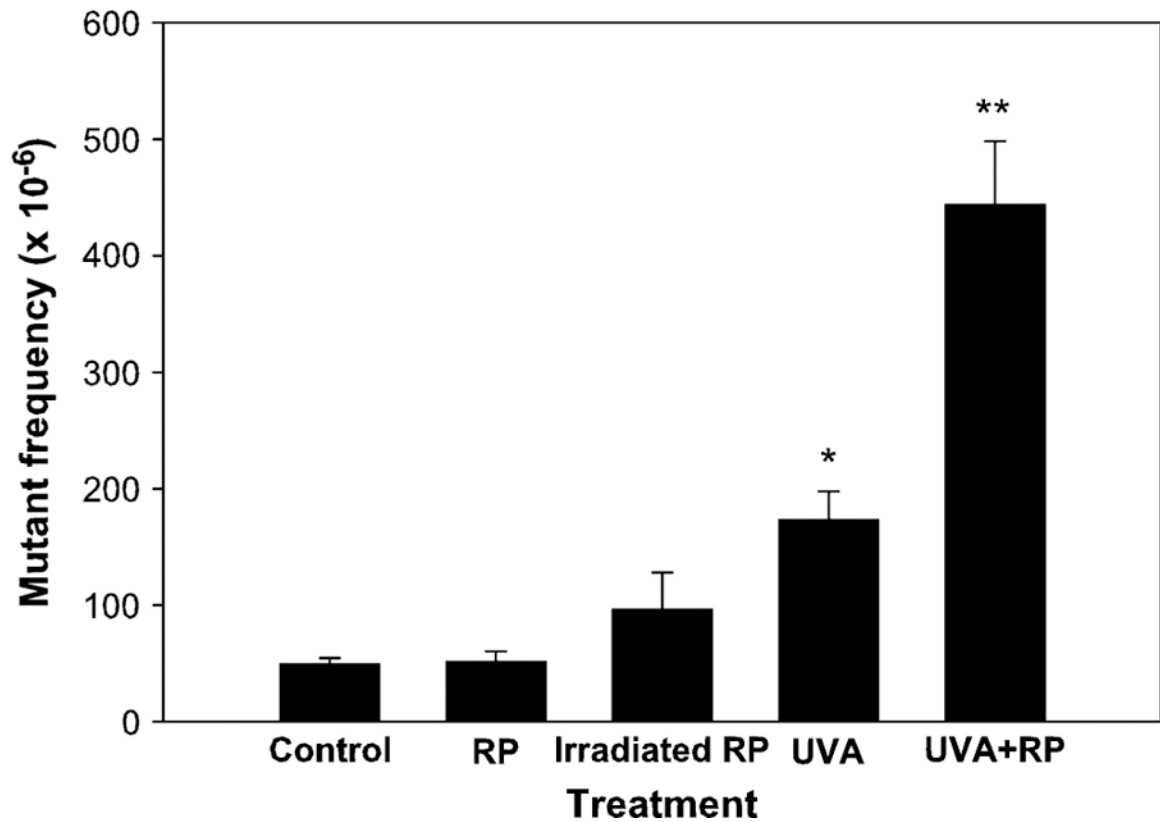
FIG. 4.

Comparison of the mutant frequencies in the *Tk* gene of mouse lymphoma cells treated with vehicle control, retinyl palmitate (RP), pre-irradiated RP, UVA, and RP + UVA. The RP concentration used for cell treatment was 25 μg/ml. The pre-irradiated RP was obtained by irradiating RP solution with 82.8 mJ/cm^2/min UVA for 30 min immediately before the cell treatment, and then the cells were treated with 25 μg/ml of the pre-irradiated RP for a 4 h incubation. For UVA and UVA + RP treatments, the cell suspensions were irradiated with 82.8 mJ/cm^2/min UVA for 30 min during the 4 h incubation. The data points represent the mean ± 1 standard deviation for three independent experiments. An asterisk indicates that the mutant frequency in this treatment is significantly different from those in control or in RP treatment groups ($p < 0.01$). Two asterisks indicate that the mutant frequency in this treatment is significantly different from those in all other groups ($p < 0.001$).
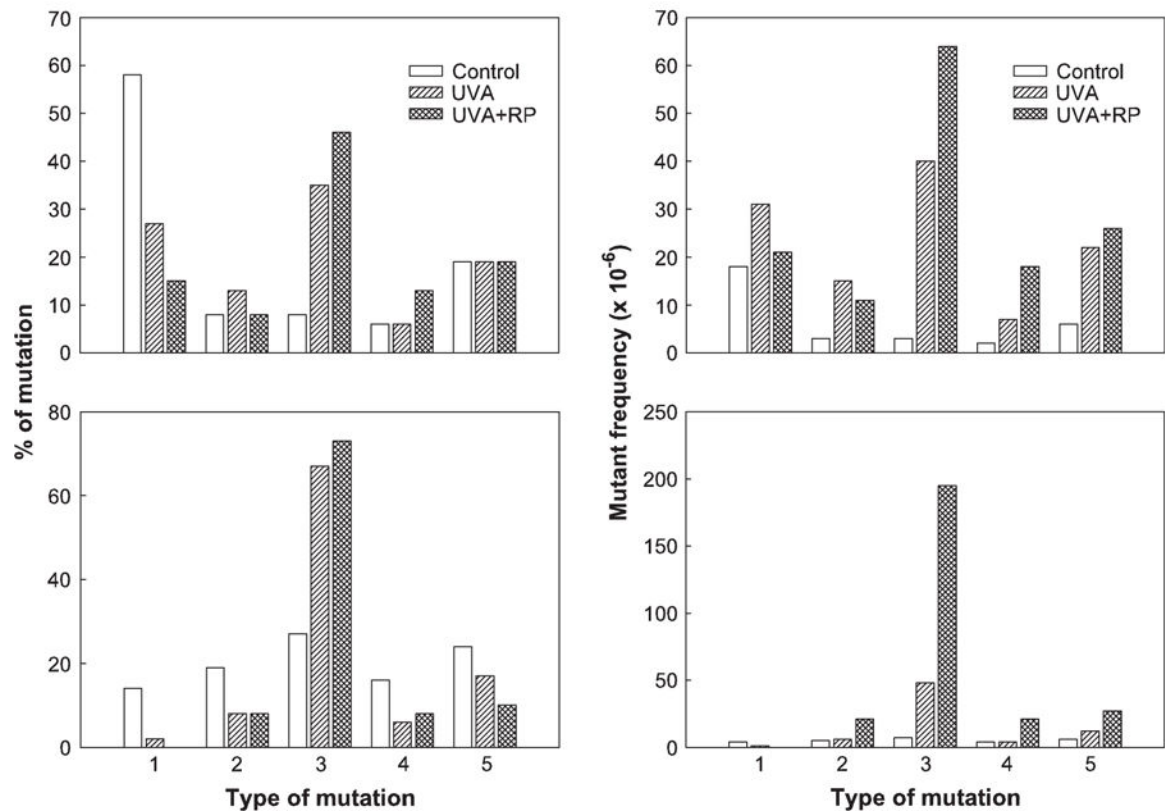
FIG. 5.
Comparison of percentage (left two panels) and mutant frequencies (right panels) of mutational types of large colonies (top panels) and small colonies (bottom panels) produced in mouse lymphoma cells treated with vehicle, UVA, and UVA + RP. The numbers indicate different types of mutations: 1, non-LOH (loss of heterozygosity); 2, LOH at *Tk* locus only; 3, LOH extending to *D11Mit42* (about 6 cM); 4, LOH extending to *D11Mit29* (about 38 cM); and 5, LOH extending to the top of chromosome 11.
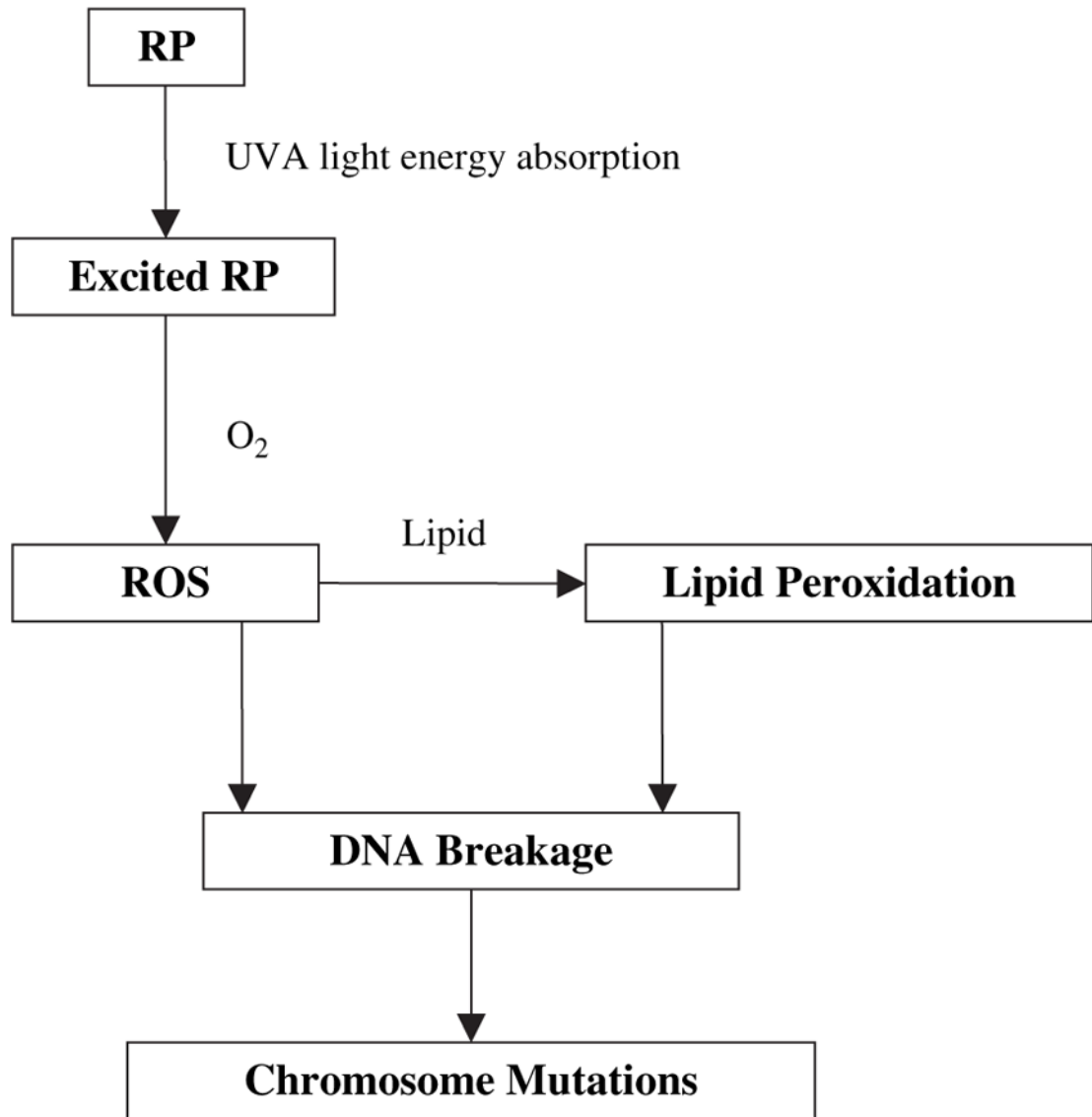
FIG. 6.
The proposed mechanistic pathway for chromosome mutations induced by combined exposure of RP and UVA in cells.

TABLE 1

Toxicity and Mutagenicity of RP, UVA, and RP with UVA in L5178Y/$Tk^{+/-}$ Mouse Lymphoma Cells

Treatment	Dose	Percent plating efficiency	Total mutant count	Mutant frequency (MF, ×10⁻⁶)	Relative total growth (%)	MF of small colonies (×10⁻⁶)	MF of large colonies (×10⁻⁶)
Control[a]	1% DMSO	84	35	57	100	26	31
RP	25 µg/ml	105	35	46	98	18	27
	50 µg/ml	92	27	40	89	26	13
	100 µg/ml	96	33	47	77	28	18
UVA[b]	5 min	110	50	63	78	29	34
	10 min	85	55	91	67	46	45
	15 min	88	76	125	63	61	64
	30 min	94	113	186	52	71	115
	45 min	96	156	270	43	125	145
RP + UVA[c]	1 µg/ml	95	111	180	75	70	110
	2.5 µg/ml	100	115	179	72	81	98
	5 µg/ml	103	151	243	68	137	106
	10 µg/ml	92	156	283	36	183	100
	25 µg/ml	70	175	437	12	287	150
4-NQO[d]	0.1 µg/ml	58	246	921	25	483	438

[a] In the control group, cells were treated with dimethyl sulfoxide (DMSO) only.

[b] Cells received 82.8 mJ/cm²/min UVA irradiation for different periods.

[c] Cells were concomitantly exposed to different concentrations of RP and 82.8 mJ/cm²/min UVA irradiation for 30 min.

[d] 4-NQO was used as a positive control.

TABLE 2

Statistical Pairwise Multiple Comparison of Mutational Spectra from Control Vehicle, UVA Alone, and RP + UVA-Treated Mouse Lymphoma Cells

Comparison	p Value	Statistically significant difference
Control versus UVA (large colony)	0.013	Yes
Control versus RP + UVA (large colony)	0.001	Yes
UVA versus RP + UVA (large colony)	0.448	No
Control versus UVA (small colony)	0.003	Yes
Control versus RP + UVA (small colony)	0.001	Yes
UVA versus RP + UVA (small colony)	0.816	No