Cytotoxicity and Mutagenicity of UVB Assessed Using Cultured Rat Fibroblast

Nobutake Akiyama 1, Satoru Murata 2, David B. Alexander 1, Hideo Yaoita 2, Yasunobu Aoki 3, and Makoto Noda 1

A retroviral vector carrying both positive (neo) and negative (herpes simplex virus thymidine kinase or HSV-tk) selection markers was constructed as a substrate for mutational assay in mammalian cells. Using a population of rat fibroblast cells carrying a single copy per cell of retroviral DNA randomly integrated in their chromosomes, we examined the cytotoxic and mutagenic activities of ultraviolet light (UV) at four wavelengths (254, 290, 300, and 320 nm). The action spectra for these activities are similar to some of the previously reported spectra for photochemical DNA modifications, erythema, cell killing, and mouse skin carcinogenesis, except at 290 and 320 nm. At 290 nm, no significant mutagenicity was observed. At 320 nm, both cytotoxic and mutagenic activities were 10 times higher than the values expected from the absorption spectrum for DNA and the action spectrum for bacterial inactivation and mutagenesis. Structural comparison of some of the HSV-tk mutants obtained after irradiation with 300 and 320 nm UV revealed partially different patterns of mutation specificity, suggesting the involvement of multiple molecular mechanisms in the genotoxicity associated with this range of UV.


UVB, cytotoxicity, mutagenicity

HISTORICAL BACKGROUND

Various assay systems to assess the risk of environmental carcinogens have been developed [for a review see ref. 1]. In one type of assays, cultured cells are used to detect inactivation of certain marker genes after treatment with mutagens, and the nature of mutations are molecularly analyzed. Markers used for such purpose include both endogenous cellular genes and genes introduced exogenously. The X-chromosome-linked hypoxanthine-guanine phosphoribosyltransferase (HGPRT) gene is frequently used as an endogenous marker, since there is only one copy of active gene per diploid cell and its inactivation is readily detected by reverse selection with an antimetabolite 6-thioguanine (6-TG). Such endogenous genes, however, are usually not suitable for molecular analyses because of their large sizes: e.g. HGPRT gene spans 44 kb 2. To circumvent this limitation, cells transfected with a "shuttle vector" carrying bacterial markers have been used [for a review see ref. 3]. Following mutagenesis, inactivation of these markers are detected by transforming individual plasmids into bacterial cells and testing their phenotypes. Such an approach makes it possible to systematically analyze the specificity of mutations induced by certain mutagens mostly in pri mate cells. One drawback to such approach, however, is the difficulty in discriminating mutations induced by mutagenesis from those induced during other experimental steps, such as transfection and plasmid recovery.

To solve these problems, Ashman and coworkers 4, 5 used retroviral shuttle vector carrying bacterial xanthine-guanine phosphoribosyltransferase (Eco-gpt) gene 4, along with another dominant selection marker (neo), and the origins of replication from bacterial plasmid as well as simian virus 40. When this retrovirus is infected into an HGPRT-deficient mouse fibroblast cell line (A9), the cells become resistant to HAT as well as

1Department of Molecular Oncology, Kyoto University Graduate School of Medicine, Yoshida-Konoe-cho, Sakyo-ku, Kyoto, 606-8601 Japan.
2Department of Dermatology, Jichi Medical School, Minamikawachi, Kawachigun, Tochigi, 329-0498 Japan.
3National Institute for Environmental Studies, Onogawa, Tsukuba, Ibaraki, 305-0053 Japan.
Address for correspondence: Department of Molecular Oncology, Kyoto University Graduate School of Medicine, Yoshida-Konoe-cho, Sakyo-ku, Kyoto, 606-8601 Japan.
DESIGN OF LTK-15 RETROVIRAL VECTOR

Herpes simplex virus thymidine kinase (HSV-tk) is capable of converting anti-herpes produgs, such as acyclovir (ACV) and ganciclovir (GCV) to a toxic metabolites, and therefore makes the host cells sensitive to this drug. HSV-tk provides a unique opportunity in which one can confer the sensitivity, rather than resistance, to a certain drug via gene transfer techniques. If the introduced HSV-tk gene is inactivated or lost, the cells become resistant to these drugs \(^8\), and this allows one to detect the gene defects in a positive manner. This marker has been widely and successfully used as a negative selection marker in gene targeting experiments.

We adapted this principle for mutational assay by constructing a retroviral vector carrying two markers, HSV-tk and neo \(^8\). When a pool of rat fibroblast cells (CREF) previously infected with this virus (named LTK-15) and selected with G418 were exposed to certain mutagen, such as UV, a dose-dependent increase in the frequency of ACV-resistant colonies were observed. It was also possible with this vector to discriminate between small mutations and large deletions simply by testing the intactness of the linked second marker, neo, in the ACV-resistant cell clones. This retroviral vector has an important advantage, because it does not require host cells pre-tagged with specific genetic markers for reverse selection, which makes it feasible to use this system in a wide variety of cells.

EVALUATION OF THE ASSAY SYSTEM \(^9\)

The HAT-selection for Eco-gpt marker/HGPRT--host system assures the intactness of the marker gene prior to mutagenesis. Since our assay system lacks such direct forward selection, we initially anticipated relatively high levels of background ACV-resistant colonies. However, even with a pooled population of LTK 15-infected, G418-selected cells, we could detect the increase in the number of ACV-resistant colonies well above background (3.5 ± 2.3 per 10⁵ cells, N = 21) after mutagenesis. These background colonies seem to arise by deletions of various sizes inside or including HSV-tk gene. Frequency of background ACV-resistant colonies did not appreciably increase even after 5 month passage of LTK15/CREF cells in G418-containing medium. Prolonged passage of the cells in the absence of G418, however, resulted in the increase of background colonies, and in this case, loss of whole viral genome was observed.

Treatment of the cells with low doses of concord, which is known to induce chromosome nondysjunction, was found to increase the frequency of ACV-resistant, G418-sensitive colonies which represent large deletions, suggesting that this system can indeed discriminate between small mutations and large deletions including the loss of whole chromosomes.

PREVIOUS STUDIES ON UV MUTAGENESIS

It is now clear that solar ultraviolet (UV) radiation in the UVB range (280-320 nm) plays major roles in non-melanoma skin carcinogenesis \(^10\) and cataract formation \(^11\)-\(^13\). Since stratospheric ozone depletion due to environmental pollution results in an increase of UVB reaching the earth’s surface \(^14\), understanding the properties of this range of UV is important for human health. Although extensive efforts had been made to obtain action spectra for photochemical DNA modifications \(^15\)-\(^18\), cytotoxicity and mutagenicity in cultured cells \(^15\), \(^19\)-\(^20\) and induction of erythema in human skin \(^22\)-\(^25\) and carcinoma in mice \(^30\), \(^31\), information on the structural specificity of mutations induced by monochromatic UVB in mammalian cells is still limited.

Specificity of UV-induced mutations in mammalian cells have been studied mainly using lac \(^I \)\(^30\), supF \(^33\), \(^34\), apry \(^35\), and p53 \(^36\), \(^37\). Apparent specificity of mutations detected by phenotypic changes of the host cells can be greatly influenced by the functional organization as well as sequence composition of the target gene employed. Thus, it is important to compare data obtained with a wide variety of target genes. Furthermore, a large fraction of previous studies employed light sources with relatively broad emission spectra. Although the actual solar beam contains UV with a wide variety of wavelengths, information on the effects of UV at various wavelengths would provide an important basis for the understanding of the interac-
tions among light of different wavelengths.

One method previously used to study UV-induced mutations is to irradiate a purified "shuttle vector" DNA which is then passaged through mammalian cells and finally recovered into bacteria. This method allows the efficient identification of a large collection of UV-induced mutants\(^33,38,39\). Although the retrovirus-based systems are not as efficient as such shuttle vector systems, they have their own advantages: e.g. minimum false positives that may occur during the passage and recovery of shuttle vectors and an ability to detect large deletions which would disable shuttle vectors. Furthermore, these retroviral systems provide a unique opportunity for us to detect mutations in a transcriptionally active gene integrated at various sites of mammalian chromosomes.

**UVB-INDUCED CELL KILLING AND MUTATIONS DETECTED WITH THE LTK-15/CREFSYSTEM**

The emission spectra for the light sources we used (290, 300, and 320 nm) were confined within a relatively narrow range (about 10 nm±indicated wavelength) in each case\(^40\). A regular germicidal lamp was used as a control. We first determined the dose-response curves for the cytotoxicity and mutagenicity using LTK15/CREF cells, and obtained action spectra. In the case of cytotoxicity, the reciprocal of the energy required to kill 90% of the cells were plotted (Fig. 1, circle). In the case of mutagenicity, the reciprocal of the energy required to obtain 1 × 10\(^{-4}\) mutation frequency was plotted (Fig. 1, triangle). These curves represent the efficiency of the light at each wavelength to induce cell killing or mutations. Interestingly, these data are consistent with the slopes of the DNA spectrum\(^10\), and the cytotoxicity measured on bacteria\(^16\) with a few exceptions as follows.

The relative cytotoxicity and mutagenicity per quantum at 320 nm were about 10 fold higher than those measured on bacteria. Most previous studies, in which action spectra of UV on mammalian cells or whole animals have been determined, employed wavelengths up to 315 nm. In the limited number of studies using wider ranges of UV, shoulders of action spectra in the wavelength range longer than 320 nm have been commonly observed. Such cases include spectra for erythema\(^10\) and cytotoxicity on human lymphoblastoid cells\(^22\), P3 human epithelial cells\(^25\) and rabbit lens epithelial cells\(^26\). Our action spectra for cell killing and the mutagenicity measured with HSV-tk gene\(^40\) fit with these data. The obvious deviation from the DNA absorption spectrum suggests the possibility of a contribution by some mechanism(s) other than direct photo-absorption by the DNA.

At 290 nm, mutagenicity was very low, and a linear dose-response curve could not be obtained\(^40\). Jones et al.\(^25\) reported similar phenomenon, albeit at much longer wavelengths: that is, mutagenicity as measured by thioguanine-resistance in P3 human epithelial cells was undetectable at 334 nm but detectable at a longer wavelength, 365 nm. Mutagenicity detected in assays using living cells is expected to be influenced by many factors, including the ability of the agent to damage cells and DNA and the ability of the cells to protect DNA from damage or to repair it. Wavelengths which yield particularly low mutagenicity in these assays may reflect photochemical properties of key molecules working in such processes.

![Figure 1](image-url). Action spectrum for cytotoxicity (○) and mutagenicity (△). The solid line represents the DNA spectrum\(^10\).
STRUCTURES OF THE MUTANTS

In our assay system, the nature of mutations can be assessed at two levels: by testing the sensitivity of colonies to G418 and by direct sequencing. By the first approach, it was found that 300 nm UV induces relatively large deletions at higher frequencies. Although the generality and mechanism of this phenomenon is presently unknown, it may have some relevance to the finding by Miguel and Tyrrell that 315 nm UV causes a significant yield of single-strand breaks in DNA. Determination of the exact deletion sites in our mutants may yield further information on the mechanism. Nevertheless, this phenomenon seems to be wavelength-dependent. It may be of interest to examine whether such deletion-inducing activity is even stronger at 290 nm, because such high frequency deletions may offer another explanation why 290 nm UV was found to be cytotoxic and yet non-mutagenic.

Sequence analyses of some of the mutant HSV-tk genes generated by 300 and 320 nm UV revealed that a majority of the mutations (34 out of 45) were the C to T transition and tandem substitutions mostly at di- or oligo-cytidines sites. Although tandem double mutations are thought to be a hallmark of UV-induced mutation, the frequencies of such mutations we observed after UVB irradiation were significantly higher than those detected previously with UVC by other workers. Reid and Loeb reported that reactive oxygen species have the ability to induce tandem double CC>TT mutations. Our observations may therefore suggest the contribution of oxygen free radicals or related molecular species in these mutational events. Transition type single base substitution at the 3'-side of dipyrimidines is also common in UV mutagenesis. Our data indicated that the 3'-side of di-cytidine sequence appears to be the most prevalent site for substitutions. This is consistent with the contribution of (6-4) photoproduct; this photoproduct forms most often at CC and TC sequences and the 3' base cannot pair correctly in this conformation. Some fraction (6 out of 21) of the C -> T transition mutations are associated with CpG sequence and may also be explain by (methyl-) cytosine deamination.

Besides these hallmark mutations, some complex mutations which cannot be explained by simple mechanisms were detected at 320 nm. Examples of such mutations are shown in Fig. 2. Some of these complex mutations (e.g. Fig.2 a) and d)) may be explained by a mechanism analogous to the error-prone DNA synthesis in bacteria; others may represent multiple events which may or may not have occurred independently. Whether such complex mutations are specific to the UV of longer wavelengths remains to be established. Such information, if confirmed, may be important, because as Setlow et al. have recently suggested based on experiments with hybrid fish, solar UVA (>320 nm) probably accounts for a majority of human melanoma cases. It is also interesting to note that Drobetsky et al. found a marked increase of T > G transversion in the aprt locus after exposure to UVA of relatively broad emission spectrum (>350 nm), as we found a similar tendency even at 320 nm and this mutation cannot be simply explained by known mechanisms.

CONCLUSIONS

In this study, we found that biological activities of UV at different wavelength differ not only in potency but also in quality. The molecular bases for such differences may be an interesting subject for future studies. Validity and usefulness of the present assay system have been confirmed through this study. Similar studies using other types of cells (e.g. skin keratinocytes which may be more relevant for UV risk assessment) and other types of mutagens may provide important

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Figure 2. Complex mutations found after 320 nm UV irradiation.
information on the mechanisms of mutagenesis and carcinogenesis.

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