Effects of the order of exposure to a binary mixture of mutagens on the induced mutation spectra in the supF gene

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We have shown previously that UVC irradiation of benzo[a]pyrene diol epoxide (BPDE)-adducted DNA (BPDE/UVC) induces an increase in mutation frequency in the supF gene greater than the calculated additive value derived from either treatment alone, with a greater absolute increase in the level of BPDE signature transversions. Possible explanations were that (i) the BPDE adducts are photoactivated to a more mutagenic lesion or (ii) the presence of UV-induced DNA damage enhanced the mutagenicity of BPDE adducts elsewhere on the DNA. In the present study, to determine which of these mechanisms is responsible for the enhanced mutagenicity of the combined treatment, plasmid pSP189 containing supF was treated with UVC radiation before BPDE treatment (UVC/BPDE). If BPDE adducts were being modified by UV irradiation to more mutagenic species, then reversing the order of exposure would be predicted to lower the mutation frequency and the number of transversions. Conversely, if merely the presence of UV damage influences the mutagenicity of BPDE adducts (or vice versa), the observed mutagenicity should be independent of the order of exposure. Previously, treatment with BPDE/UVC increased the mutation frequency by >400% over the calculated additive value derived from the individual BPDE and UV exposures. In the present study, treatment with UVC followed by BPDE increased the mutation frequency by only ~60%, compared with the corresponding calculated additive value. However, whilst this shows that the order of treatment affects the mutation frequency, there was little change in the percentage of base substitutions in the two spectra. Hence, whilst the change in mutation frequency is consistent with UVC directly enhancing the mutagenicity of the BPDE adducts, the similarity in the types of mutations induced by BPDE/UVC and UVC/BPDE suggests that the mechanism may not be that simple.

Introduction

In vivo, DNA is subject to a variety of damaging mutational insults from both exogenous and endogenous sources. DNA can become modified by the introduction of miscoding or non-instructional DNA adducts, which can result in the formation of mutations when the DNA is replicated. The nature of the induced mutation and the distribution of mutations within the target gene (the mutation spectrum) is dependent on a number of factors, including the nature of the induced damage, the efficiency of repair of the damage, the polymerase involved in replicating the DNA and the sequence context in which the damage occurs (Brash et al., 1987; Levy et al., 1992, 1996; Courtemanche and Anderson, 1994; Bigger et al., 2000; Canella and Seidman, 2000; Seo et al., 2000). This understanding has been derived from large numbers of studies of single mutagens. However, many exposures in vivo occur as complex mixtures or combinations of mutagens, such as those found in airborne or waterborne pollution (Houk, 1992; Deflora et al., 1993; White et al., 1996; Viskari et al., 1997; Rank et al., 2001). Little work has been carried out to date on such combinations. Using the Salmonella reversion assay, DeMarini and co-workers have examined mutations induced by a number of complex mixtures and some binary mixtures (DeMarini, 1998) and have shown that the mutation spectra tend to be dominated by mutations induced by a small number of mutagens within the complex mixture. This is consistent with evidence from studies of mutations in the p53 gene of human tumours, in which the observed mutation spectra have been linked with specific mutagen exposure (Hussain and Harris, 2000).

In our laboratories we are interested in whether DNA damage from one mutagen can interact with a second mutagen, so influencing the resulting mutation spectra. We have previously used the supF mutation assay (Seidman et al., 1985; Parris and Seidman, 1992) to investigate mutations induced by a combination of benzo[a]pyrene diol epoxide (BPDE) adducts and UV irradiation (Routledge et al., 2001) and found that UV irradiation of target DNA that had first been adducted with BPDE (BPDE/UVC) led to an enhancement in mutation frequency that was more than additive of the individual BPDE and UV exposures. We had chosen BPDE and UV as mutagens because they give rise to predominantly different mutations, allowing for the possibility of assigning particular mutations to one or other of the mutagens in the binary mixture. Examination of the mutation spectrum induced by the combined treatment showed that although UV signature transition substitution mutations were prevalent, there was a greater absolute increase in the level of BPDE signature transversions. We speculated that possible explanations were (i) that UV irradiation of the BPDE adducts led to a modification of these adducts to more mutagenic lesions or (ii) that the presence of UV damage enhanced the mutagenicity of BPDE adducts elsewhere on the DNA. If the BPDE adducts were being modified by UV irradiation, then reversing the order of exposure (UV followed by BPDE) would be predicted to lower the mutation frequency and alter the profile of induced

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et al. fetal calf serum (Life Technologies, Paisley, UK) at 37 °C were grown in Dulbecco’s modified Eagle’s medium supplemented with 10% horse serum provided by Dr A.Dipple (National Cancer Institute, Frederick, MD) and (National Institute of Aging, National Institute of Health, Baltimore, MD).

### Materials, shuttle vector plasmid, bacterial strain and cell line

All chemicals were from Sigma (Poole, UK) unless otherwise stated. The plasmid pSP189 containing the supF gene (Parris and Seidman, 1992; Seidman et al., 1985) and Escherichia coli strain MBM7070 were gifts from M.Seidman (National Institute of Aging, National Institute of Health, Baltimore, MD). Human embryonic adenovirus-transformed kidney cells (Ad293) were provided by Dr A.Dipple (National Cancer Institute, Frederick, MD) and were grown in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum (Life Technologies, Paisley, UK) at 37°C in 5% CO₂ in air.

### Treatment of DNA with UVC and BPDE

The pSP189 shuttle vector was treated with UVC (254 nm) radiation at an intensity of 0.7 mW/cm² for 1.5 min (giving a dose of 0.63 kJ/m²). Plasmid was further treated with BPDE (~10 μM) in aceton, as described previously (Routledge et al., 2001). Control plasmid reactions with solvent only, UVC only and BPDE only were also performed. After removal of excess BPDE with methanol (95%), plasmids were precipitated with 10 μL 3 M sodium acetate (pH 5.5) and 10 μL isopropyl alcohol. Aliquots of plasmid were used to transform electrocompetent MBM7070 (Escherichia coli strain MBM7070) to remove any unreplicated DNA. Aliquots of recovered plasmid were used to transform electrocompetent MBM7070 E.coli by electroporation using Gene Pulser apparatus (Bio-Rad, Hercules, CA). Transformants were plated onto LB agar plates containing ampicillin (100 μg/ml) and X-gal containing medium, whereas wild-type colonies were blue.

### Results

Table I shows the mutation frequencies for the UVC/BPDE exposure in comparison to the previously published mutation frequency for the BPDE/UVC exposure (Routledge et al., 2001); also shown are the mutation frequencies of the controls, the BPDE and UVC exposures alone and the calculated additive value derived from the individual BPDE and UV exposures for the respective studies. In the present study, the relative increase in mutation frequency for UVC/BPDE was only ~60% greater than the calculated additive value derived from the individual BPDE and UV exposures, compared with the corresponding >400% increase noted for BPDE/UVC in the previous study (Routledge et al., 2001). Furthermore, it was generally noted for the combined treatments that UVC/BPDE induces a much lower difference in mutation frequency in comparison to the controls, BPDE treatments alone and UVC irradiations alone (Table I). BPDE followed by UVC (Routledge et al., 2001) induces an increased mutation frequency 657-, 16- and 7-fold greater than the control.
BPDE treatment alone and UVC irradiation alone, respectively, whereas UVC followed by BPDE (the present study) induces a mutation frequency increase only 59-, 7- and 2-fold greater than the control, BPDE treatment alone and UVC irradiation alone, respectively.

One hundred and seven mutants obtained from the UVC/BPDE experiment were sequenced and the types of mutations are shown in Table II. Table II shows that there is little difference between the two combined treatments in terms of the types of mutations that were induced. The majority were in the form of single base substitutions, with a few tandem and multiple substitutions and frameshifts. The proportion of multiple mutations decreased slightly compared with BPDE/UVC whilst frameshifts increased slightly compared with BPDE/UVC. The types of base substitution mutations are shown in Table III, with the mutation spectrum being illustrated in Figure 1. Included in Tables II and III and Figure 1 are the mutation spectra previously obtained for BPDE treatment alone, UVC irradiation alone and BPDE/UVC (Routledge et al., 2001) for comparison with the new spectrum.

The GC→AT transition, followed by the GC→TA transversion, were the major mutations for both combined BPDE and UVC treatments. All other possible base substitutions were detected except the AT→CG transversion, which interestingly was only induced once (in the UVC/BPDE multiple spectrum) throughout both experiments (Routledge et al., 2001). Table III also shows the types of mutations observed as multiple mutations (plasmids with more than one mutation within the target gene). The major multiple base substitution was the GC→AT transition in both combined treatments, but there is an overall preference for transversions when BPDE is administered second. For UVC/BPDE a total of 9/107 plasmids sequenced (8.4%) contained multiple mutations compared with 12/69 (17%) for BPDE/UVC (previously reported; Routledge et al., 2001). Multiple mutations are thought to be derived via a different mechanism to single mutations, possibly involving strand break repair (Seidman et al., 1987). Thus the increase in multiple mutations for BPDE/UVC is consistent with the increase in strand breaks previously observed when BPDE-adducted DNA was UV irradiated compared with the irradiation of unadducted DNA (Routledge et al., 2001). As before (Routledge et al., 2001), the spectrum of multiple mutations

Fig. 1. Mutation spectra depicting single base and tandem substitutions induced by the various treatments used. The 5’→3’ sequence of the transcribed strand of the wild-type supF gene is shown, with letters below the wild-type sequence indicating the position and type of point mutations induced by the various treatments. BPDE, UVC and BPDE/UVC spectra from Routledge et al. (2001).
differed from that of single mutations (Figures 1 and 2). The Cariello Hyperg program for comparing mutation spectra (Cariello et al., 1994) was used to determine that the spectra of mutations produced from the BPDE/UVC and UVC/BPDE treatments shown in Figures 1 and 2 are significantly different ($P < 0.05$). In the mutation spectra containing single base substitutions (Figure 1), hotspots of mutation were observed at positions 110, 115 and 175 in the BPDE only spectrum, 104, 123, 156 and 172 in the UVC only spectrum, 110, (124 borderline hotspot), 155, 163, 172 and 175 in the BPDE/UVC spectrum (Routledge et al., 2001) and 124, 156 and 172 in the UVC/BPDE spectrum. In the multiple mutation spectra there were mutation hotspots at positions 156 and 172 after BPDE/UVC treatment and at 139 after UVC/BPDE treatment (Figure 2).

**Discussion**

In our previous study the UV irradiation of pSP189 DNA that had been adducted with BPDE gave rise to a synergistic enhancement of mutations compared with the individual BPDE and UV treatments. The purpose of reversing the order of exposure was to determine whether it was necessary for the BPDE adducts to be already present on the DNA in order for the enhancement of mutation frequency in the combined exposure to be observed. The results show that whilst there is an increase in mutation frequency for UVC/BPDE compared with the calculated addition of mutation frequency of UVC and BPDE, it is much lower than when the DNA was adducted with BPDE prior to irradiation. We had proposed two possible hypotheses: (i) that UV irradiation of the BPDE adducts led to a modification of these adducts to more mutagenic lesions or (ii) that the presence of UV damage enhanced the mutagenicity of BPDE adducts elsewhere on the DNA. The lower mutation frequency of the reversed treatment (UVC/BPDE) in this study supports the hypothesis that UV irradiation of the BPDE adducts produces a modified adduct that was more mutagenic. However, when considering the relative contribution of different types of base substitution mutations to the two mutation spectra (Table III), the fact that these are very similar in the two combination spectra, regardless of the order of treatment, argues against the UV-induced modification of BPDE adducts.

The other source of information from this type of study is the distribution of mutations along the \textit{supF} gene. Within any induced mutation spectrum there are sites which are more commonly mutated than others, which are referred to as hotspots of mutation. There are two ways to compare these hotspots. Firstly, if the hotspots [hotspots were assumed when the number of mutations observed was \(\geq 4\)-fold greater than the number expected for a random (Poisson) distribution] in the mutation spectra shown in Figure 1 are compared, it can be...
seen that there are certain sites that are hotspots for the different spectra. For example sites 110 and 115 are hotspots in the BPDE spectrum but not in the UVC spectrum, whereas site 156 is a hotspot in the UVC spectrum but not the BPDE spectrum. For the combination spectra, some hotspots occur at sites of hotspots in the two individual spectra, whereas other sites appear as hotspots that are not hotspots in the individual spectra (e.g. site 155 in the BPDE/UVC spectrum and site 124 in the UVC/BPDE spectrum).

Alternatively, it can be argued that comparing hotspots between different spectra in this way is not valid because it does not take into account the difference in mutation frequency for the different treatments. Table IV summarizes the mutation frequency at each individual site, where more than one mutation was recorded, in the supF gene for all four spectra. This takes into account both the number of mutations observed at a site and the mutation frequency for that treatment. Hence, there is very little difference between the mutation frequency at site 156 in the UVC spectrum (4.2 in 10^4) compared with the same site in the BPDE/UVC spectrum (6.6 in 10^4), but there is a more marked difference between the mutation frequency in these two spectra at site 123 (2.1 in 10^4 versus 9.9 in 10^4). From Table IV, the sites that show the biggest difference in mutation frequency in the combined treatments versus the predicted mutation frequency for that site from the two individual treatments are sites 110, 115, 123, 124, 155, 163, 172, 175 and 178 for the BPDE/UVC spectrum and sites 110, 124, 155 and 172 for the UV/BPDE spectrum. The increase in mutation frequency between the BPDE/UVC and UVC/BPDE treatments is most notable at sites 110, 155, 163, 172, 175 and 178. It is not clear, however, what conclusions can be drawn from this analysis, other than to observe that the reversal of treatment has altered the induced mutation frequency in the target gene.

The strongest hotspot in the BPDE/UVC treatment is site 155, which has a mutation frequency of 33 in 10^4, compared with 4.4 in 10^4 at the same site in the UVC/BPDE treatment (note that identical mutations have arisen independently in different cells, as confirmed by checking that the signature sequence of pSP189 differs for mutants with identical mutations). What has caused this dramatic difference and why should this site be such a strong hotspot compared with site 156, as these sites occur in an eight base palindromic region? It has previously been noted that site 156 always occurs as a hotspot in UV-induced mutation spectra, whereas the presence of a UV-induced hotspot at site 155 depends on the experimental conditions (Canella and Seidman, 2000). Our results fit into this pattern in that site 156 is a hotspot when UV treatment occurs independently or prior to BPDE adduction, but site 155 is a hotspot when the DNA has been adducted first. As we have not performed a polymerase stop assay or ligation-mediated PCR assay to locate hotspots of DNA damage in our experimental treatments we cannot say if this effect is related to the preferential distribution of BPDE damage at this site.

This study was carried out to examine whether the order of mutagen exposure of BPDE and UVC in a binary treatment altered the induced mutation frequency and mutation spectrum. The results show that whilst the types of mutations induced were similar, both the frequency of mutation and the distribution of mutations along the target gene did change when the order of mutagen exposure was reversed. The nature of the results support the suggestion that the mutagenicity of BPDE adducts is enhanced by UV irradiation, but as the relative proportion of different types of mutations in the two treatments was similar it is not clear by what mechanism this enhancement has occurred.

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References

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