

Double-Strand Break Yield Following ^{125}I Decay

Effects of DNA Conformation

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The decay of iodine-125 (^{125}I) is accompanied by the emission of low-energy electrons that dissipate most of their energy in approximately 10 nm from the decay site. In mammalian cells, the $\cdot\text{OH}$ generated by these electrons are also confined to a small volume. Iodine-125 is thus an excellent probe for assessing the radiobiologic effects produced by $\cdot\text{OH}$ in close proximity to the site of a decaying atom. We have compared in pUC19 plasmids (naked DNA) and in Chinese hamster V79 lung fibroblasts (chromatin) the modulation by the $\cdot\text{OH}$ scavenger dimethyl sulfoxide (DMSO) of ^{125}I -induced DNA double-strand breaks (DSB). The data indicate that DMSO cannot protect plasmid DNA against DSB damage from ^{125}I decaying within a few angstroms from DNA. However, DMSO attenuated DSB production in V79 cells following the decay of DNA-incorporated ^{125}I , thus suggesting that chromatin structure fosters some DSB formation by indirect mechanism(s). DSB production depends on the environment and/or conformation of DNA. Consequently, current biophysical modeling of DNA damage that is based on naked and non-compacted DNA is inadequate for explaining radiobiologic effects at the cellular level.

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Iodine-125 (^{125}I) decays by electron capture (EC) and internal conversion (IC). During these processes, a shower of ~ 21 extremely low-energy (< 1 keV) Auger, Coster-Kronig, and super Coster-Kronig electrons occurs (1–4). As the bulk of the energy of these low-energy electrons is dissipated within a few nanometers from the decay site, dosimetric calculations have predicted that their biologic effects depend critically on the proximity of the radionuclide to DNA. Experimental results in synthetic oligonucleotides (5), phage DNA (6, 7), and plasmids (8, 9) have confirmed these expectations and have shown that within a few angstroms of *such naked DNA molecules* each decay of ^{125}I produces one double-strand break (DSB). The same is observed in intact phage which do not contain histones and other nucleoproteins (7, 10). *In mammalian cells*, however, our recent in vitro results have shown an increase in cell survival (11) and a concomitant decrease in the yield of DSB (12) after the intranuclear decay of ^{125}I in the presence of the $\cdot\text{OH}$ scavenger dimethyl sulfoxide (DMSO). These findings indicate that (i) the radiobiologic effects are caused mainly by indirect mechanism(s) and (ii) *more than one* DSB is produced in chromatin per ^{125}I decay. Since DMSO does not affect the DSB yield in plasmid DNA exposed to ^{125}I decay, i.e. one DSB is

produced per decay of ^{125}I (13), we thought it likely that the differences in DNA conformation between chromatin and naked DNA would lead to the greater contribution of scavengable $\cdot\text{OH}$ in the former instance (12). In an attempt to clarify these seemingly contradictory results (DSB are scavengable in chromatin but not in naked DNA), we have investigated further the effects of scavengers on DSB formation in Chinese hamster V79 cells following the accumulation of DNA-incorporated ^{125}I decays in frozen cells and have compared our results with those obtained following the decay of this Auger electron emitter in close proximity to pUC19 plasmid DNA (9, 13).

MATERIAL AND METHODS

Radiochemicals

5- ^{125}I iodo-2'-deoxyuridine ($^{125}\text{IUdR}$) was purchased from DuPont NEN Research Products (specific activity 81 TBq/mmol). After cell uptake and DNA incorporation of this thymidine analog, the ^{125}I atom is positioned within the double-stranded DNA helix (see Table 1). [^{125}I]iodoHoechst 33342 (^{125}IH) was synthesized from trimethylstannylHoechst 33342 (9). This iodinated analog of the DNA minor-groove-binding agent Hoechst 33342 has an equilibrium association constant (K_a) of 2.6×10^7

M^{-1} (14) and, after DNA binding, it positions the ^{125}I atom within $\sim 4 \text{ \AA}$ from DNA (9, 13).

pUC19 plasmids: [^{125}I]odoHoechst 33342 studies

pUC19 plasmids (2686 base pairs in length, MW 1.7×10^6) were grown in *E. coli* (DH5 α) bacteria at 37°C in TB medium containing thiamine (10 $\mu\text{g/ml}$) and ampicillin (150 $\mu\text{g/ml}$). Following amplification (42 h) in the presence of ^3H -thymidine (1.48 MBq/ml, 248 GBq/mmol; DuPont NEN Research Products), the plasmids were purified using a resin column (Qiagen Incorporated). The procedure leads to the production of >90% pure supercoiled (SC) plasmid DNA. The plasmid yield was determined fluorometrically in the presence of ethidium bromide (0.5 $\mu\text{g/ml}$) using calf thymus DNA as a standard.

Measurement of DNA double-strand breaks in pUC19 plasmids

Tritium-labeled pUC19 plasmids (10 μg) were placed in 100 μl 10 mM sodium phosphate buffer, pH 7.4, containing $^{125}\text{I}\text{H}$ ($\sim 1.85\text{--}22.2 \text{ MBq}$) in the presence or absence of 0.2 M DMSO. The samples were stored at 4°C to accumulate radiation dose. At various time intervals (up to 35 days), the plasmid-DNA samples were resolved by gel electrophoresis (0.8% agarose) into supercoiled (SC), nicked (N), and linear (L) forms, representing undamaged DNA, SSB (single-strand breaks), and DSB, respectively. The DNA-containing bands were excised, dissolved in 0.5 M HCl, and the concentration of ^3H -DNA was determined in a liquid scintillation counter (the samples were counted twice at a 6-week interval to correct for ^{125}I spillover). DSB yields were calculated using the method of Cowan et al. (15) as described by Kassis et al. (9).

Chinese hamster V79 lung fibroblasts: 5-[^{125}I]odo-2'-deoxyuridine studies

Chinese hamster V79 lung fibroblasts were cultured in vitro in Dulbecco's Modified Eagle Medium (GIBCO) with 5% fetal bovine serum (FBS, GIBCO), 0.5 ml penicillin-streptomycin (Sigma), and 0.2 ml gentamicin (Sigma) per 100 ml. Following a 4-h incubation at 37°C, $^{125}\text{I}\text{UdR}$ was added and the cells were re-incubated for 18 h (control cells were labeled with ^{14}C -thymidine [^{14}C -TdR]). The cells were then washed with phosphate-buffered saline, trypsinized, spun down, and re-suspended ($\sim 10^6$ cells/ml) in ice-cold medium containing one of the

following: (i) 5% FBS, (ii) 5% FBS and 10% DMSO, (iii) 10% FBS, or (iv) 10% FBS and 15.6% DMSO. A portion of these cells was used to determine DNA-incorporated ^{125}I activity after trichloroacetic acid precipitation (16). The remaining cells were frozen (-135°C or -196°C) and, following ^{125}I decay, the number of decays (N) accumulated by cells stored for a given time was calculated according to the equation:

$$N = A_{18} \left(1 - e^{-\ln 2 * \frac{t}{T_{1/2}}} \right) * \frac{T_{1/2}}{\ln 2} \quad [1]$$

where A_{18} is the content of ^{125}I per cell, t is the time of storage, and $T_{1/2}$ for ^{125}I is 60.14 days.

Measurement of DNA double-strand breaks in V79 cells

Neutral elution (17) was performed after the accumulation of various numbers of ^{125}I decays. Briefly, nucleopore polycarbonate filters (2 μm , Costar Scientific Corporation) were placed in funnels and washed with 5 ml elution buffer (0.2% SDS, 0.05 M Tris, 0.05 M glycine, 0.025 M EDTA, pH 9.6). The funnels were loaded with 4 ml lysing solution (0.2% SDS, 0.05 M Tris, 0.05 M glycine, 0.025 M EDTA, pH 9.6, containing 0.5 mg proteinase K per ml) followed by 2.5×10^5 cells in 0.25 ml. Pumping (3 ml/h) was started 15 min later, the collection of 3-ml fractions was initiated, and after an hour 20 ml elution buffer was added. The volume of each fraction was determined and its radioactive content assessed in a Packard Auto-Gamma 500 counter (the radioactivity of the filters was also measured). Finally, the funnels were washed with 10 ml 0.4 N NaOH, and the radioactive content of 1-ml aliquots was measured. DNA elution profiles of ^{14}C -TdR-labeled controls were determined using a liquid scintillation counter. The fraction of radioactivity retained on each filter after elution with 11 ml (F11 value) was calculated, and this was used to obtain the differential rate of DNA elution for irradiated (*irr*) compared with unirradiated (*ctr*) cells (18). This value, called strand scission factor (SSF), is defined as:

$$\text{SSF} = -\log_{10} \left(\frac{\text{F11}_{irr}}{\text{F11}_{ctr}} \right) \quad [2]$$

RESULTS

DSB in pUC19 plasmid DNA after [^{125}I]odoHoechst 33342 exposure

Inspection of the gels from $^{125}\text{I}\text{H}$ -plasmid incubation mixtures at early time points shows the simultaneous appearance of the nicked and linear forms, the latter indicating the early production of DSB (Fig. 1). At later time points, no fluorescent bands are seen within the gels, suggesting the complete fragmentation of the plasmid DNA. Addition of the $\cdot\text{OH}$ scavenger DMSO has no effect on the visual appearance/disappearance of the L bands, i.e. on the production of DSB and the subsequent fragmentation of DNA by the minor-groove binder. The increased intensity

Table 1

Characteristics of Auger-electron-emitting compounds

	$^{125}\text{I}\text{UdR}$	$^{125}\text{I}\text{H}$
Analog of	Thymidine	Hoechst 33342
Position in DNA	Base substitution	Minor groove
DNA binding	Covalent	Hydrogen bonds
^{125}I decay position	Within DNA helix	$\sim 4 \text{ \AA}$ from DNA

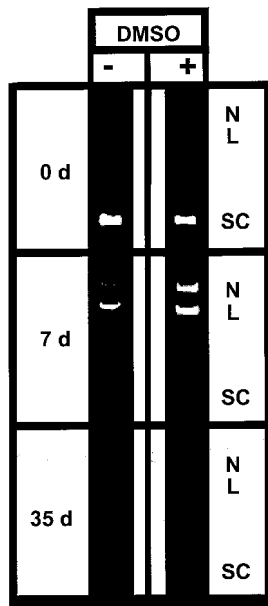


Fig. 1. Agarose gels of pUC19 plasmid DNA after exposure to ^{125}I \pm 0.2 M DMSO. SC, supercoiled DNA; N, nicked DNA (= SSB); L, linear DNA (= DSB). Intensity of L DNA band is same in both presence and absence of DMSO. Note also total disappearance of UV-visible bands on day 35 indicating fragmentation of DNA (13).

of the nicked DNA band indicates that DMSO protects against SSB, as has been observed by others (19).

Quantitative analysis confirms these findings. The yield of DNA DSB per ^{125}I decay both in the presence and absence of DMSO is 0.90 ± 0.11 (13). The number of DSB produced per decay of ^{125}I diminishes at higher doses, consequent to the fragmentation of these DNA molecules.

DSB in V79 cell nuclear DNA after $5\text{-}^3\text{H}$ -thymidine exposure

Elution profiles of ^{14}C -TdR-labeled control V79 cells frozen in the presence or absence of DMSO are similar, thus demonstrating that the freezing of mammalian cells in the absence of a cryoprotectant, i.e. in culture medium alone, does not lead to DSB formation (20). The elution data for DNA immediately after the 18-h incubation of cells with ^{125}I UdR is similar to that of controls that do not contain ^{125}I UdR. For cells accumulating decays from DNA-incorporated ^{125}I , the DNA elutes at faster rates with increasing doses. When the SSF are calculated and plotted as a function of ^{125}I decays (Fig. 2), the DSB yields are found (i) highest in 5% serum alone; (ii) decreased in 10% serum; (iii) still lower in 10% or 15.6% DMSO plus 5% or 10% FBS.

DISCUSSION

We and others have been examining the radiobiologic effects of Auger electron irradiation in various biologic test

systems. In studies that assess the radiotoxicity of DNA-incorporated ^{125}I (11, 16, 21–25), an observed exponential reduction in clonal survival has been attributed to the localized deposition of energy in the immediate vicinity of the decaying atom (16, 24–27). Since these findings imply that the radiobiologic effects originating from the decay of Auger-electron-emitting radionuclides are a consequence of direct action, we and others throughout the 1970s and 1980s believed that indirect mechanisms do not contribute to the results observed in mammalian cells.

In the early 1990s, Rao et al. (28–30) reported that the co-injection of very small amounts of the well-known radioprotectant cysteamine (MEA) into the testes of mice strongly mitigated the biologic effects of ^{125}I decaying within the DNA (^{125}I UdR) or the cytoplasm (^{125}I -labeled N,N,N' -trimethyl- N' -(2-hydroxyl-3-methyl-5-iodobenzyl)-1,3-propanediamine [$\text{H}^{125}\text{IPDM}$]) and concluded that 'the Auger effect may be due largely to the indirect action of radical species produced by the shower of low-energy Auger electrons emitted by the radionuclide' (29). More recent results from the same laboratories have reiterated these findings. For example, Nara and co-workers (31) found that vitamin C protects from radiation damage caused by both DNA-incorporated ^{125}I and cytoplasmically localized ^{125}I . Similar enhancement of spermhead survival has been observed with vitamin A and soybean oil (32) and with DMSO (33). Unfortunately, credence in the conclusion reached in all of these in vivo studies (i.e. the Auger effect is largely indirect) was weakened in light of the experimental system and conditions used.

The first issue concerned the dose of radioprotectant. To avoid toxicity, the investigators injected extremely small quantities of each radioprotectant. For example, Rao et al. (28, 29) injected $0.75 \mu\text{g}$ MEA into the testes ($\sim 0.1 \text{ g}$) 4 h prior to the injection of ^{125}I UdR or $\text{H}^{125}\text{IPDM}$. Similarly, Goddu et al. (33) injected mice with $0.3 \mu\text{g}$ DMSO and 4 h later with ^{125}I UdR. Since the MW of these radioprotectants is low, their residence time within the testes will be very short, particularly in comparison with the very long biologic half-time of ^{125}I UdR (once incorporated into the DNA of cells, the radionuclide is indefinitely retained). Consequently, these agents are likely to be present during a very small fraction of the total accumulated ^{125}I decays.

The second issue involved the effective concentrations of the radioprotectants within the testes. As these were much lower than those necessary to confer radioprotection, the reduction in radiotoxicity observed cannot be due to the injection of these agents. For example, radiobiologic studies in various mammalian cell lines have demonstrated repeatedly that DMSO provides no protection at $< 0.5\%$ concentration (60 mM). In one of the studies (33), the maximum DMSO concentration that could have been reached in the testes model immediately post-injection is

0.06 mM. It is difficult to understand how such a low DMSO concentration could have scavenged the $\cdot\text{OH}$ generated by the decaying ^{125}I atoms.

Hofer & Bao (34) examined the effects of the radioprotector cysteamine on cell death and micronucleus formation after the decay of ^{125}I in CHO cells frozen in the presence of 1 M DMSO and concluded that the cryoprotectant *could not* protect these mammalian cells against the high-LET-like biologic effects of DNA-incorporated ^{125}I . Howell et al. (35) were also unable to protect Chinese hamster V79 cells in vitro against the toxicity of DNA-incorporated ^{125}I by storing the cells in 5% DMSO at 11°C. Our studies (11, 36), however, have consistently shown that mammalian cell survival after exposure to ^{125}I decays at 0°C or in the frozen state is enhanced in the presence of the $\cdot\text{OH}$ scavenger DMSO. These in vitro results are the first to demonstrate that the radiotoxicity of Auger electrons from ^{125}I decay in mammalian cells is caused mainly ($\geq 80\%$) by indirect mechanism(s). Most recently, Bishayee et al. (37) reported that 10% DMSO at 11°C is in fact radioprotective.

As an accompaniment to the cytotoxicity experiments, we have also examined the possible radioprotective effects of DMSO and serum on DSB formation after the decay of ^{125}I in close proximity to mammalian cell nuclear DNA (12, present work) and plasmid DNA (9, 13). The induction of DSB in mammalian cell DNA is attenuated in the presence of DMSO (12), in parallel with the enhanced cell survival in the presence of this agent (11). Taken together with our present results (Fig. 2), in which the yield of DNA DSB is reduced by the presence of DMSO and/or higher serum concentration during accumulation of ^{125}I decays, it is clear that indirect mechanism(s) underlie DSB production following the decay of ^{125}I in V79 cell nuclear DNA. The fact that DSB production is prevented by DMSO compels us to conclude that in the absence of this

radical scavenger *more than one DSB is produced per decay of ^{125}I* . In addition, the present results with serum suggest that the actual number of DSB per ^{125}I decay in cellular DNA depends on the scavenging conditions.

In contrast to the results obtained in mammalian cells, our studies with plasmid DNA indicate that the same DSB yields (0.90 ± 0.11 DSB/ ^{125}I decay) are obtained in the presence and absence of DMSO (13). Thus, for such naked DNA molecules, the direct mechanism predominates. Similar conclusions have also been reached by Kandaiya and co-workers (19) and by Panyutin & Neumann (38) who examined the radioprotective effects of DMSO in short synthetic oligonucleotides. While the absence of radioprotection seen in plasmids and oligonucleotides seems to be at odds with the substantial radioprotection observed in intact mammalian cell nuclear DNA, we believe that these results can be explained (12) when the packaging of DNA in the cell nucleus is taken into consideration. Short, synthetic oligonucleotide fragments or circular naked plasmid DNA are in a stable B form double helix, with 10.5 base pairs per helical turn, a major groove and a minor groove. Within a mammalian cell nucleus, however, these rod-like linear structures (39) do not exist. Within chromatin, DNA is tightly packed. A decaying ^{125}I atom may damage by direct action adjacent DNA sites and the cluster of $\cdot\text{OH}$ produced may attack sites that are many nucleotides (e.g. 100s or 1000s of base pairs) away from the atom but placed in close proximity to it by DNA supercoiling (12). Furthermore, in the absence of compaction (e.g. in plasmids), the distances separating the loops of double-stranded DNA are large and ^{125}I decay can only cause DSB in the base pairs immediately adjacent to the ^{125}I atom (direct action).

CONCLUSIONS

1. In naked DNA (e.g. synthetic oligonucleotides, plasmids), the number of DSB induced per ^{125}I decay is one; in the absence of DNA supercompaction, DSB are induced by direct mechanism(s), which at high doses lead to DNA fragmentation.
2. Chromatin structure (highly packed DNA) provides conditions for the formation of more than one DSB per ^{125}I decay by indirect mechanism(s).
3. Radical scavengers, such as FBS and DMSO, reduce the yields of DSB in chromatin.
4. DSB production depends on the conformation and/or environment of DNA; biophysical modeling of DNA damage that is based on naked and/or non-compacted DNA is inadequate for explaining radiobiologic effects at the cellular level.
5. The radiotoxicity of Auger electrons from ^{125}I decay in mammalian cells is caused mainly ($\geq 80\%$) by indirect mechanism(s).

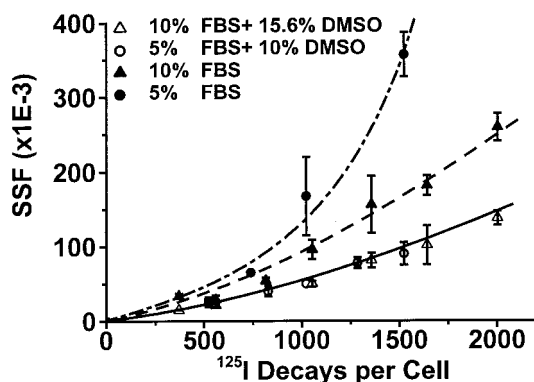


Fig. 2. Induction of DSB in V79 cell nuclear DNA by decays of ^{125}I accumulated in frozen state. Strand scission factor (SSF), which is indicator of DNA DSB, is plotted as function of ^{125}I decays per cell. Note that data for 5% and 10% FBS + 10% and 15.6% DMSO can be fitted by single curve.

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