Radiotoxicity of Iodine-125-Labeled Oligodeoxyribonucleotides in Mammalian Cells

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We investigated the distribution, stability and radiotoxicity of ^{125}I-oligodeoxyribonucleotides (^{125}I-ODN) in human fibrosarcoma HT-1080 cells to study the radiotoxic effects of the Auger electron emitter ^{125}I delivered to the cells by ODN. **Methods:** We delivered ^{125}I-ODN into the cells via complexing with a liposomal delivery system. To assess the intracellular distribution and stability of ^{125}I-ODN delivered by the liposomal delivery system, we used autoradiography, fluorescent and confocal microscopy and electrophoresis. To study the radiotoxicity of the unbound ^{125}I-ODN, we used a clonogenic assay. The radiotoxicity of ^{125}I-ODN delivered by the liposomal delivery system was compared with that of freely diffusible ^{125}I-antipyrine, membrane-excluded ^{125}I-bovine serum albumin and DNA incorporated ^{125}I-deoxyuridine (^{125}I-UdR). **Results:** Oligodeoxyribonucleotides accumulated in the cell nucleus within a few hours of incubation. On the basis of the number of decays at 37% survival, ^{125}I-ODN are 2 times more radiotoxic than ^{125}I-antipyrine, which is freely diffusible into cells, and 8 times more radiotoxic than ^{125}I-bovine serum albumin, which remains outside cells. However, the radiotoxicity of unbound ^{125}I-ODN is almost 3 orders of magnitude lower than that of DNA-incorporated ^{125}I-UdR. The ^{125}I-ODN are not significantly degraded by intracellular nucleases during the time of uptake incubation. **Conclusion:** The dramatic difference in radiotoxicity between ^{125}I-ODN and ^{125}I-UdR confirms that, despite the nuclear localization, ^{125}I-ODN are not bound to or incorporated within the genomic DNA. Our data demonstrate that the radiotoxicity of Auger electron emitters is determined by the radiation dose delivered to nuclear DNA, not necessarily to the nucleus. Therefore, relatively high intracellular concentrations of unbound ^{125}I-ODN can be achieved without causing significant cell death.

Key Words: iodine-125; oligodeoxyribonucleotides; radiotoxicity; triplex; liposomal delivery system; liposomes

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Synthetic oligodeoxyribonucleotides (ODN) have drawn a great deal of attention in recent years as a tool for manipulation of gene expression (*1,2*). They localize mainly in the nucleus on delivery into a cell (*3*). To affect the expression of a targeted gene, ODN are designed to bind to either the RNA (antisense approach) or the DNA (antigene approach) of this gene. In the latter case, the ODN must be able to form a triple helix (or triplex) with the target DNA duplex. In the triplex, the third strand is located in the major groove of the DNA duplex and forms Hoogsteen hydrogen bonds with the bases of the duplex (*1*). Adenine and thymine of the triplex-forming ODN recognize the A-T base pair, and cytosine and guanine recognize the C-G base pair of the duplex. The specificity of triplex-forming ODN-DNA recognition is very high and is comparable with that of complementary strands pairing in the Watson–Crick duplex. Therefore, if there is no sequence-specific target in the genome for an ODN, it will not be able to bind to genomic DNA via triplex formation.

If radiolabeled, ODN may usefully carry radioactivity to specific DNA or RNA sequences in targeted cells or tissues. The possibility of using ODN labeled with Auger electron emitters as imaging agents and radiopharmaceuticals has been discussed (*4,5*).

We propose an approach for targeting specific sites in the genome (genes) that combines the sequence-specific action of ODN with localized damage produced by decay of Auger electron emitters such as ^{125}I. We called this approach gene radiotherapy. In vitro studies demonstrated that the decay of DNA-incorporated or DNA-bound ^{125}I causes DNA double-strand breaks (DSB) (*6,7*). The DSB are localized within 10 bp around the site of decay and occur with an efficiency close to one break per decay (*8,9*). Because DSB produced by the decay of the ^{125}I are highly mutagenic (*10,11*), these findings make ODN very promising agents for gene inactivation via sequence-specific breakage of DNA inside the cells.

In addition to the sequence-specific action, ^{125}I-ODN are radiotoxic, like any other radioactive compound, due to an overall radiation dose delivered to the cell. Therefore, it is important to know the limits of the amount of ^{125}I-ODN that could be delivered into the cells without causing death from nonspecific irradiation. However, the radiotoxicity of Auger electron emitter labeled ODN has not been studied.

The radiotoxicity of ^{125}I-labeled compounds dramatically depends on their intracellular localization (*12–16*). The relative biological effectiveness varies from 7.3 for DNA-incorporated ^{125}I-deoxyuridine (^{125}I-UdR) through 4.5 for the DNA-bound intercalating drug ^{125}I-iodoproflavine and down to 1.3 for cytoplasm-localized ^{125}I-iododihydrorhodamine (*12*). The cytocidal effect of the DNA-incorporated or DNA-bound ^{125}I is similar to that of high linear energy transfer radiation, whereas the effect of extranuclear localized ^{125}I is similar to that of low linear energy transfer radiation (*17*). This positional effect is characteristic of ^{125}I and other Auger electron emitters and can be explained by two facts: first, the primary target for ionizing radiation is nuclear DNA; and second, the radiotoxicity of Auger electron emitters is mainly due to low energy electrons with very short ranges (1–10 nm). If such decays occur in close proximity to DNA, the combined action of the Auger electrons results in molecular damage to DNA comparable with that produced by high linear energy transfer radiation (*17*).

Herein, we studied the radiotoxicity of ^{125}I-ODN delivered into human cells with liposomes to facilitate cellular penetration and overcome biodegradability. Liposome-assisted delivery has been successfully used in numerous studies using both the antisense and the antigene approach (*3,18–22*). We have used a liposome delivery system (LDS) that has been shown to be efficient for gene delivery in cell cultures and after intravenous injection in mice (*23*). To assess the intracellular distribution and stability of ^{125}I-ODN delivered by LDS, we used autora-

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diography, fluorescent and confocal microscopy and electrophoresis. To study the radiotoxicity of the ^{125}I-ODN, we used a clonogenic assay. The radiotoxicity of ^{125}I-ODN delivered by LDS was compared with that of freely diffusible ^{125}I-antipyrine, membrane-excluded ^{125}I-bovine serum albumin (^{125}I-BSA) and DNA-incorporated ^{125}I-UdR.

MATERIALS AND METHODS

Labeled Compounds

Iodine-125-deoxyuridine (7.4×10^{13} Bq/mmol), ^{125}I-antipyrine (1.7×10^{11} Bq/mmol) and ^{125}I-BSA (3.7×10^{7} Bq/mg) were purchased from ICN Biomedical (Costa Mesa, CA); ^{125}I-dCTP (8.1×10^{13} Bq/mmol) was obtained from DuPont–New England Nuclear (Boston, MA).

ODN was synthesized on an ABI-394 DNA synthesizer (Applied Biosystems, Foster City, CA) followed by purification from polyacrylamide gel (PAG), as previously described (*9*). The template oligonucleotide was biotinylated using BioTEG modifiers (Glen Research, Sterling, VA). The ODN were labeled with ^{125}I using DNA polymerase and ^{125}I-dCTP by the primer extension method as described previously (*9*).

Fluorescein-labeled ODN (FITC-ODN; 500 μM) were obtained from Epoch Pharmaceuticals (Bothell, WA).

Oligodeoxyribonucleotides/Liposome Delivery System Complex

The LDS liposomes were prepared according to the described procedure (*22,23*). The liposomes composition was dioctadecylamidoglycylspermine:1,2-dioleoyl-sn-glycero-3-phosphatidylethanolamine (0.6:1). The initial concentration of the LDS was 4.8 mg lipid/ml. We used a working concentration of 12 μg/ml lipid. One hundred microliters of the LDS liposome solution were mixed with 100 ng (24 μCi) of ^{125}I-ODN dissolved in 100 μl of 150 mM NaCl. The mixture was left for at least 1 hr at room temperature before further use. Incubation of the DNA/liposomes mixture at room temperature increased the uptake of the ODN/LDS complex.

Cell Line and Culture Conditions

The human fibrosarcoma HT-1080 cell line (American Type Culture Collection, Manassas, VA; CCL-121) was used (the doubling time is 26 hr, the average diameter is 20 μm). The cells were grown in monolayer in Eagle's minimum essential medium (EMEM) supplemented with 0.1 mM nonessential amino acids, 50 μg/ml penicillin-streptomycin and 10% heat-inactivated fetal bovine serum (FBS), purchased from BioWhittaker (Walkersville, MD). All procedures were performed according to American Type Culture Collection recommendations.

Cell Treatment with Iodine-125

Cells grown to confluence were treated with trypsin, dispensed into sterile tubes, collected by centrifugation at $500 \times g$ for 10 min and resuspended in 3 ml of EMEM medium to a final concentration of 5×10^{6} cells/ml. Three 1-ml aliquots were prepared, 24 μCi of the ^{125}I-ODN/LDS complex were added to the cells in each of two tubes and the tubes were placed into a CO_2 incubator. The cells in the third tube were incubated in a CO_2 incubator without any treatment, frozen and used to determine plating efficiency.

The first tube was used to determine the uptake of ^{125}I, as previously described (*24*). Serial aliquots (100 μl) of the cell suspension were layered gently onto the surface of 200 μl FBS in 400-μl microfuge tubes at various times after incubation with the ^{125}I-ODN/LDS complex. The microfuge tubes were spun for 1 min at $12{,}000 \times g$ in a microcentrifuge and frozen immediately in an ethanol-dry ice mixture. The bottoms of the tubes containing the cell pellet were sliced with a sharp blade, and the radioactivity in the cell pellet and the supernatant medium was counted in a gamma scintillation counter (Auto-Gamma 5650, Packard, Downers Grove, IL). As a negative control, 0.6 μCi cell-free ^{125}I-ODN/LDS complex was spun through the FBS. In this case, 10% of the total radioactivity was found at the tip of the tube. This value was taken as a background. When the uptake curve determined from the first tube reached a plateau, the cells from the second test tube were divided into seven plastic vials. An equal volume of cryomedium with 15% dimethylsulfoxide (BioWhittaker) was added to each vial. The vials were set in a slow-freezing system (Nalgene, Rochester, NY) and placed at −70°C. The next day, the vials were placed in liquid nitrogen and stored to accumulate decays.

The cells at the same concentration were also treated with ^{125}I-antipyrine (27 μCi) and ^{125}I-BSA (50 μCi). After 3-min incubation, the cells were frozen as described above.

To obtain the ^{125}I-UdR survival curve, cells grown to 50% confluence in a flask were treated with 0.07 μCi ^{125}I-UdR. The cells were left in a 37°C 5% CO_2 incubator overnight (18 hr). The next day, the radioactive medium was removed and the culture flasks were rinsed twice with phosphate-buffered saline (PBS), replenished with 10 ml of complete EMEM containing 10^{-4} M thymidine and incubated again for 1 hr. The cells in the flasks were washed with PBS, trypsinized, suspended in EMEM, divided into seven plastic vials and frozen as described above. To determine ^{125}I-UdR incorporation, an aliquot of ^{125}I-UdR-treated cells was counted in the gamma counter.

Retention of Iodine-125-Oligodeoxyribonucleotides/Liposome Delivery System

HT-1080 cells were suspended in 1 ml of EMEM containing ^{125}I-ODN/LDS. After a 3-hr incubation at 37°C, cellular uptake was measured by the microfuge method (*24*).

To measure the retention of ^{125}I-ODN/LDS, cells were layered onto the surface of 2 ml of FBS, sedimented by centrifugation, resuspended in 3 ml PBS and reincubated at 37°C. Immediately after the PBS was added and at several intervals thereafter, the radioactive cell content was determined, and the percentage of retained activity was calculated and plotted against time.

Autoradiography

Samples for autoradiography were prepared from 200 μl of cell suspension, treated with ^{125}I-ODN/LDS as described above and contained approximately 10^{5} cells/ml, using a cytocentrifuge (StatSpin Cytofuge; PGC Scientifics, Gaithersburg, MD). Cell monolayers on slides were dried and fixed in 1 part acetic acid and 3 parts 96% ethanol for 45 min. Alternatively, cells were grown on slides covered with 0.1% gelatin in Petri dishes with 10 ml of EMEM. After growing for 48 hr, 24 μCi of the ^{125}I-ODN/LDS complex were added for 4 hr. The medium was removed, and the cells were fixed directly in the plate with the acetic acid-ethanol mixture.

The autoradiography procedures were performed as described previously (*25,26*). Dried samples were coated with the NTB3 photoemulsion (Eastman Kodak, Rochester, NY) in a dark room, dried and exposed in a light-tight box in a refrigerator at 5–10°C. After ^{125}I exposure, the slides were developed with Kodak D-19, fixed with Kodak rapid fixer, dried, stained with hematoxylin and eosin (Sigma, St. Louis, MO) and viewed with a light microscope (Carl Zeiss, Germany). All photoreagents were purchased from Eastman Kodak.

To assess the intracellular distribution of decays, silver grains were counted in 30 randomly selected cells. The mean numbers of grains found in nucleus and cytoplasm were calculated and used to assess the dose delivered by the labeled compounds into cells.

Fluorescent Microscopy

Cells for fluorescent analysis were grown on coverslips covered with 0.1% gelatin in six-well plates with 5 ml EMEM in each well. After growing for 48 hr, 0.5 μM FITC-ODN (Epoch Pharmaceuticals) mixed with LDS (2.5 μg ODN/8.3 μl LDS) were added. The coverslips were removed from the wells after 1, 2 and 3 hr, rinsed with medium and embedded into Vectashield mounting medium (Vector Laboratories, Burlingame, CA). Specimens were analyzed with a fluorescent microscope (Carl Zeiss).

Analysis using a laser-assisted confocal microscope was performed as described previously (*3,23*). HT-1080 cells were incubated for 2 and 3 hr with FITC-ODN/LDS at a concentration of 0.5 μM. Then cells were rinsed and collected, and cytocentrifuge samples were prepared. Cells were fixed, mounted under coverslips and analyzed with a Nikon Optiphot photomicroscope (Nikon, Melville, NY) equipped with Bio-Rad MRC-1000 laser scanning confocal imaging system (Bio-Rad, Richmond, CA).

Electrophoresis of Oligodeoxynucleotides

The frozen ^{125}I-ODN-treated samples were thawed on ice, and the cell pellet was collected by centrifugation, resuspended in 20 μl of staining solution containing formamide (U.S. Biochemical Corp., Cleveland, OH), heated to 100°C and analyzed in a 12% PAG along with control labeled ODN and labeled ODN from the supernatant. Gels were quantitated using a BAS 1500 Bio-Imaging Analyzer (Fuji, Tokyo, Japan).

Clonogenic Assay

The cells were assayed for survival by their colony-forming ability. The appropriate number of cells was plated in Petri dishes containing complete medium and incubated for 7 days until visible colonies of 50 cells or more developed (*27*). The colonies were stained with crystal violet and counted visually. Each data point represents an average count of three plates.

The survival fraction was plotted against accumulated disintegrations per cubic micron of cell suspension (for ^{125}I-ODN, ^{125}I-antipyrine and ^{125}I-BSA) and/or disintegrations per cell (for ^{125}I-ODN and ^{125}I-UdR). Survival curves were fit to a single-hit, multitarget equation using a Power Macintosh computer and KaleidaGraph software (Abelbeck Software, Reading, PA).

RESULTS

Cellular Uptake and Retention of ^{125}I-Oligodeoxynucleotides

Figure 1 shows the uptake of carrier-free ^{125}I-ODN and LDS-delivered ^{125}I-ODN into HT-1080 cells. In the case of free ^{125}I-ODN, the radioactivity associated with the cells gradually increased and reached a plateau at 5% after 300 min of incubation. In the case of LDS-assisted delivery, the maximum uptake was 35% and was reached after only 100 min of incubation. The subsequent slow decrease in cell-associated radioactivity was most likely due to intracellular degradation of ^{125}I-ODN. Therefore, the use of liposomes significantly increases and accelerates the uptake of ^{125}I-ODN.

The efflux of ^{125}I-ODN/LDS was only 4% after the first wash with PBS and reached 13% after the first hour of washing (Fig. 2). This shows that the uptake measured by sedimentation through the serum (*24*) corresponds only to the ^{125}I-ODN truly delivered inside the cells and that the loss of these ^{125}I-ODN during the washing and fixation procedures in the imaging experiments (see below) is negligible.

Distribution of Oligodeoxynucleotides Inside the Cells

Figures 3A and B show autoradiographs of the cells that were treated with ^{125}I-ODN/LDS and spread on a slide in a cytocentrifuge. Cells were stained with hematoxylin and eosin. The cytoplasm in these samples is not very well preserved and, thus,

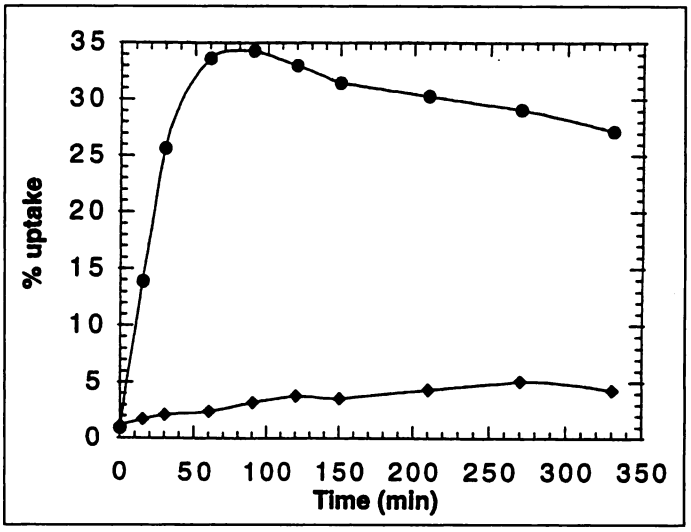

FIGURE 1. Uptake of ^{125}I-ODN by HT-1080 cells. ●, cells were treated with 24 μCi ^{125}I-ODN/LDS complex; ◆, cells were treated with ^{125}I-ODN. After the indicated time of incubation in a CO_2 atmosphere, aliquots of cell suspension (100 μl) were taken, layered onto the surface of 200 μl FBS, spun in a microcentrifuge and frozen. The radioactivity of the cell pellet was counted and presented as a percentage of the total radioactivity. Values of ODN/LDS complex uptake are corrected for radioactivity (10%) found in tips in the absence of cells. The actual average uptake per cell of ^{125}I-ODN was 0.01 Bq/cell.

appears only as a pink ring. Silver grains (black dots) correspond to the ^{125}I decays and are located mostly in the center of the cells, i.e., in their nuclei. To make certain of ODN localization, the experiment was repeated with cells grown on slides and treated with ^{125}I-ODN deposited directly on the surface of the slides (Fig. 3C and D). These cells are better seen, and their cellular outlines can be easily distinguished. The concentration of silver grains within the nuclei is also more clearly seen.

Iodine-125-deoxyuridine was localized in the nuclei of practically all treated cells, whereas ^{125}I-antipyrine and ^{125}I-BSA were distributed mostly outside the cells (data not shown). We cannot rule out the possibility that the two later compounds were washed away during processing.

The fate of the oligonucleotides inside cells after delivery with the LDS was ascertained using FITC-ODN and fluorescent microscopy. After 1-hr incubation with 0.5 μM FITC-ODN/LDS a punctate pattern of fluorescence was observed (Fig. 4A). This pattern is characteristic of the FITC-ODN concentrated in

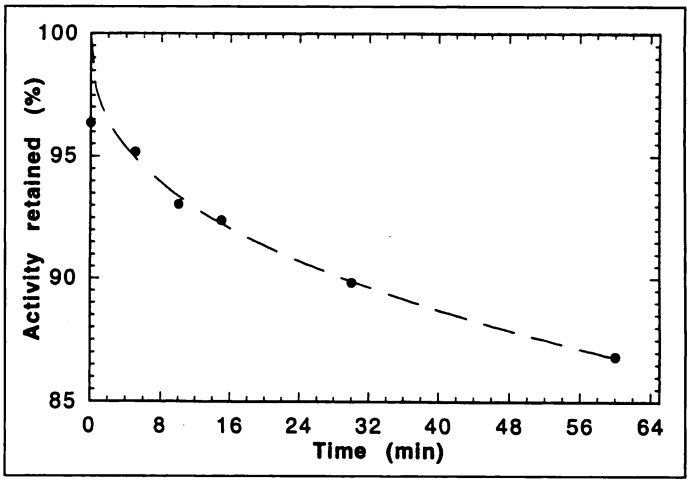

FIGURE 2. Retention of ^{125}I-ODN/LDS by HT-1080 cells as a function of incubation period at 37°C.

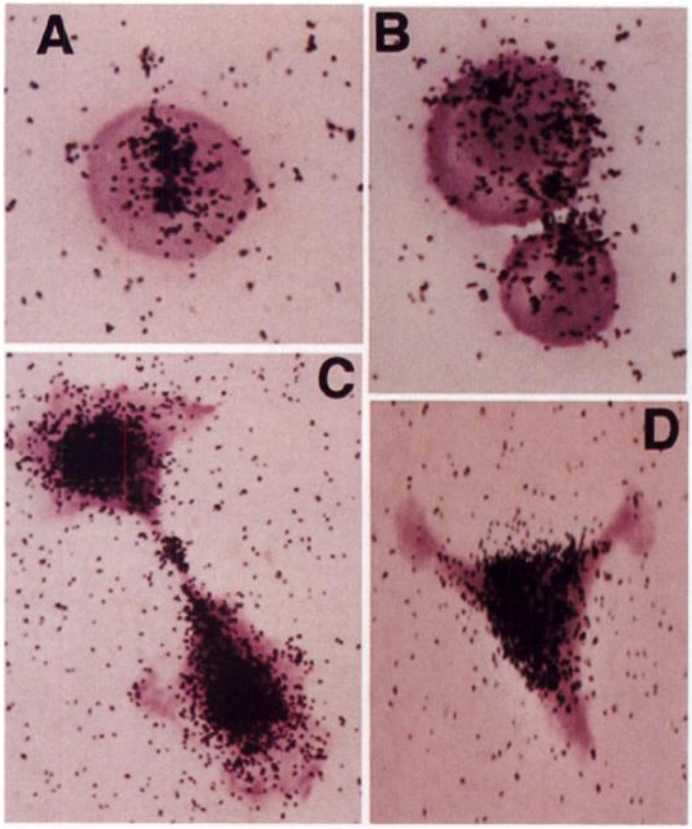

FIGURE 3. Autoradiographs of cells treated with ^{125}I-ODN and prepared in a cytocentrifuge (A and B) or grown and treated directly on the surface of a slide (C and D). Cells were stained with hematoxylin and eosin. Magnification, ×100.

endocytic vesicles (*28,29*). One hour later, along with the bright spots, a diffuse fluorescence throughout the cytoplasm and nuclei became visible (Fig. 4B). This diffuse pattern of fluorescence corresponds to the ODN that have been released from the endosomes and liposomes. After 3 hr of incubation, intense fluorescent spots are no longer observed, and most of the diffuse fluorescence is located in the nuclear compartment (Fig. 4C). These photos illustrate the mechanism of the ODN/LDS delivery: first, ODN/LDS were concentrated in endocytic vesicles; then, ODN were released from these vesicles and the liposomes into the cytoplasm; and, finally, they concentrated in the nuclei. No fluorescence was observed in the control untreated cells.

Nuclear localization of the FITC-ODN was confirmed by confocal microscopy, which allows observation of a tomographic cross-section (1 μm) of a cell with high resolution; the intracellular distribution of FITC-ODN can thus readily be appreciated. After 2 hr of incubation, fluorescence is localized in cytoplasm and nuclei (Fig. 5A); after 3 hr of incubation, fluorescence is localized in the nuclear compartment in nearly all treated cells (Fig. 5B).

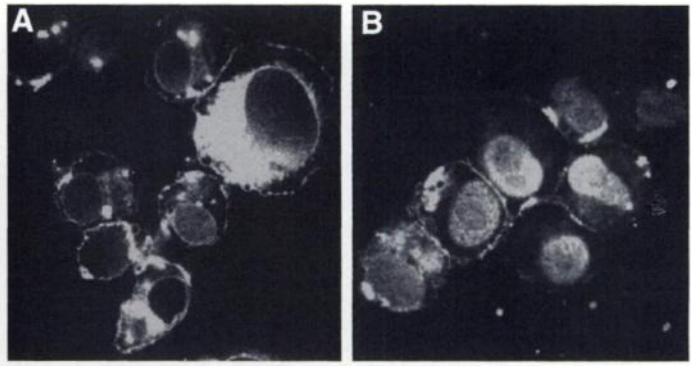

FIGURE 5. Laser-assisted confocal microscopy analysis of intracellular ODN localization after exposure to FITC-ODN/LDS. Cells incubated for 2 hr with 0.5 μM FITC-ODN/LDS (A) and cells incubated for 3 hr (B) are shown. Magnification, ×100.

Evaluation of Iodine-125-Oligodeoxynucleotides Degradation

Iodine-125-ODN was extracted from the frozen cells and analyzed by electrophoresis in a 12% PAG. They were compared with unincorporated ^{125}I-ODN extracted from the medium and with freshly labeled ^{125}I-ODN. The results after 50 days of decay accumulation are shown in Figure 6. In each lane (native ODN, ODN from cells and ODN from medium), there is a major band at the top of a ladder. The length of the major band corresponds to the full length of ^{125}I-ODN, which is 39 nucleotides [See Fig. 1 in (*9*)]. The rest of the ladder represents products of degradation of the ODN. They can be observed, since decay of ^{125}I in one of three possible locations in ^{125}I-ODN results in still radiolabeled fragments of shorter lengths. Based on the positions of deoxycytidine (^{125}I-dC) residues in ODN, the predicted lengths of these shorter fragments are 34, 26, 18 and 12 nucleotides. Maximum band intensities in the ladder correspond approximately to fragments 34, 26, 18 and 12 nucleotides long based on comparison with the markers (Fig. 6). These are exactly the lengths of the fragments produced by decay of ^{125}I. Therefore, the degradation of ^{125}I-ODN appears mainly due to decay of ^{125}I and not to the actions of intracellular nucleases during uptake or incubation.

Radiotoxicity of Iodine-125-Labeled Compounds

We used colony formation as a biological endpoint. The average plating efficiency of control untreated cells frozen for 1–50 days was 25% ± 5%. The viability of control cells obtained on the first day after freezing was assumed to represent 100% survival and was in good agreement with the plating efficiency.

The percentage survival of ^{125}I-ODN/LDS-treated cells compared with those exposed to freely diffusible ^{125}I-antipyrine and

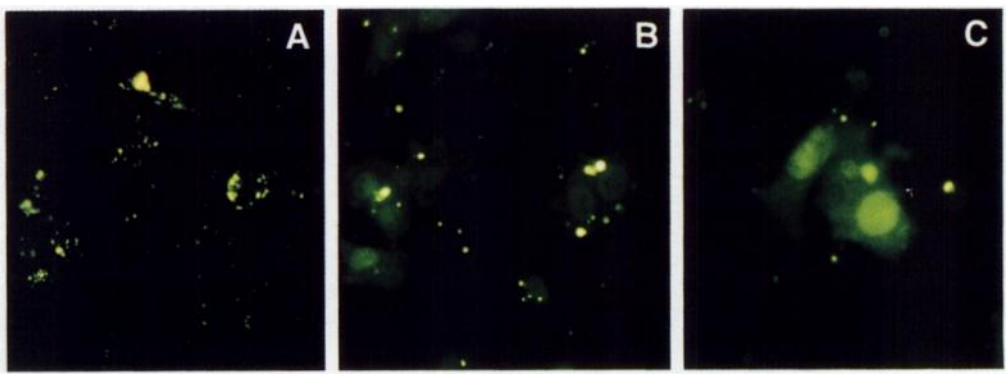

FIGURE 4. Fluorescent analysis of cells grown on gelatin-covered coverglasses and treated with 0.5 μM FITC-ODN/LDS for 1 hr (A), 2 hr (B) and 3 hr (C). Magnification, ×40.

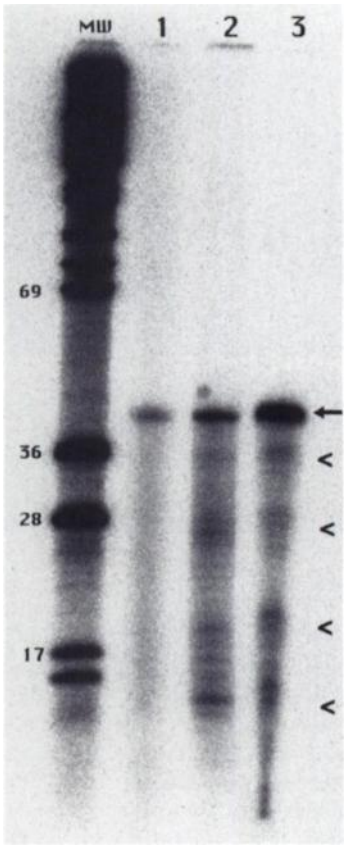

FIGURE 6. PAG analysis of the integrity of ^{125}I-ODN in frozen HT-1080 cells. MW, molecular weight markers (pUC 18 digested with *MspI* restriction enzyme). Lane 1, native ^{125}I-ODN; lane 2, ^{125}I-ODN extracted from the cells; lane 3, ^{125}I-ODN extracted from the medium. The arrow indicates the position of intact ^{125}I-ODN. Maximal intensities are marked on the right with carets.

^{125}I-BSA, which remains outside the cells, was plotted as a function of decays per unit volume (Fig. 7). Survival fractions of ^{125}I-UdR- and ^{125}I-ODN/LDS-treated cells were plotted as a function of accumulated decays per cell using the uptake values (Fig. 8).

The data in Figures 7 and 8 were fitted to a single-hit, multitarget equation (*30*):

$$S = [1 - (1 - e^{-D/D_0})^n] \times 100,$$

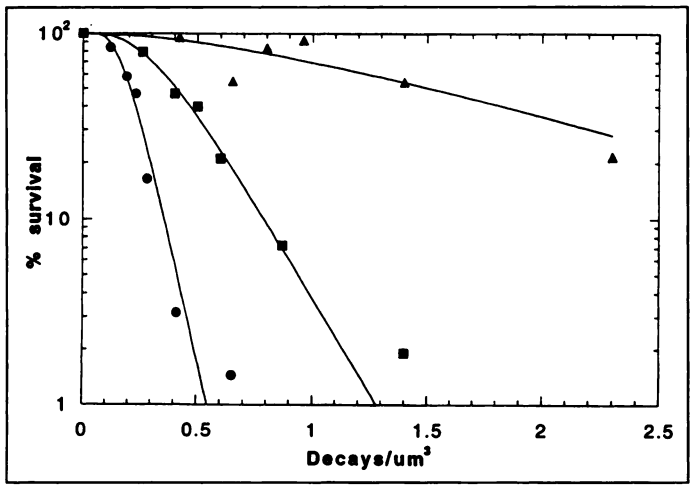

FIGURE 7. Survival curves of HT-1080 cells treated with ^{125}I-ODN/LDS (●), ^{125}I-antipyrine (■) and ^{125}I-BSA (▲). Cells in suspension were incubated with 24 μCi ^{125}I-ODN-liposomes complex for 1.5 hr and frozen. Survival was assayed by colony formation after decay accumulation for 1–50 days. Iodine-125-antipyrine (27 μCi) and ^{125}I-BSA (50 μCi) were added to the cell suspension 3 min before freezing. The data are plotted as a function of decays per unit of volume and fitted as described in the text. For ^{125}I-ODN, $D_0 = 0.08 \pm 0.01$ ($n = 8.24 \pm 3.27$); for ^{125}I-antipyrine, $D_0 = 0.21 \pm 0.02$ ($n = 4.40 \pm 0.94$); for ^{125}I-BSA, $D_0 = 1.19 \pm 0.7$ ($n = 2.10 \pm 1.55$).

where S is the percent survival and D is the number of decays per unit volume or per cell. The data were fitted using logS as the dependent variable and D_0 and n as the independent variables. The better fit to this equation is not meant to be taken as verification of a model, and no mechanistic meaning is ascribed to D_0 and n. The fitted curves are presented in Figures 7 and 8.

The interpolated 37% survival values (D_{37}) are summarized in Table 1. The D_{37} of cells treated with ^{125}I-ODN is 2 times lower than the D_{37} of cells treated with ^{125}I-antipyrine and 8 times lower than that of cells treated with ^{125}I-BSA. At the same time, Figure 8 and Table 1 show that the D_{37} of cells treated with ^{125}I-ODN is almost 3 orders of magnitude higher than that of cells treated with ^{125}I-UdR.

DISCUSSION

We have shown that ^{125}I-ODN delivered into the cells by LDS localized mainly in the nucleus, and yet the resultant radiotoxicity is almost 3 orders of magnitude less than that of DNA-incorporated ^{125}I-UdR. The nuclear accumulation of ODN was demonstrated by autoradiography and fluorescent microscopy and is in agreement with other studies of the intracellular distribution of ODN when delivered into cells (*28,29,31*). Zelphati and Szoka (*23*) have recently unambiguously shown separation of ODN from liposomes in cells and proposed a mechanism for ODN release from cationic lipids such as dioctadecylamidoglycylspermine that is present in LDS. According to their model, the ODN-liposome complex after internalization by endocytosis induces a "flip-flop" of anionic lipids from the cytoplasm-facing monolayer of the endosome membrane. This leads to displacement of ODN from the cationic lipid and their release into the cytoplasm.

The ^{125}I-ODN were not significantly degraded by intracellular nucleases during the uptake incubation (Fig. 6). This is important for interpreting the survival data, since the ^{125}I-dC released via degradation could be incorporated into genomic DNA and later cause additional DNA damage. In addition, we could not detect any radioactivity associated with genomic DNA purified from ^{125}I-ODN-treated cells (data not shown). Nevertheless, the contribution of such secondary incorporation cannot be completely ruled out, because this may occur after plating the cells for the survival assay. In interpreting our data, we disregarded the fact that the ODN that we used can potentially form a triplex with a single target per genome. The expected effect of such triplex formation would be equivalent at best to incorporation of just one ^{125}I-UdR per entire genome and would not result in any noticeable changes in the survival curve.

Our D_{37} values presented in Table 1 are in good agreement with previously published data (*13,32,33*), taking into account the different cell lines and assay conditions used in the cited reports. The fact that ^{125}I-ODN are considerably more radiotoxic than ^{125}I-BSA and more radiotoxic than ^{125}I-antipyrine is because of the nuclear localization of the former. The dramatic difference in radiotoxicity between ^{125}I-ODN and ^{125}I-UdR confirms that despite the nuclear localization, ODN are not bound to or incorporated within the genomic DNA. In this sense, ODN are possibly the first studied ^{125}I-labeled compounds that localize within the nucleus and yet do not bind to the DNA.

Using conventional dosimetry, we estimated the total energy delivered to the cell nucleus by D_{37} decays, assuming that each decay in the nucleus delivers 1.8 fJ/decay, decay in the cytoplasm delivers 0.14 fJ/decay and decay outside the cell delivers 0.5×10^{-9} fJ/decay (*15,32,34*). In the case of

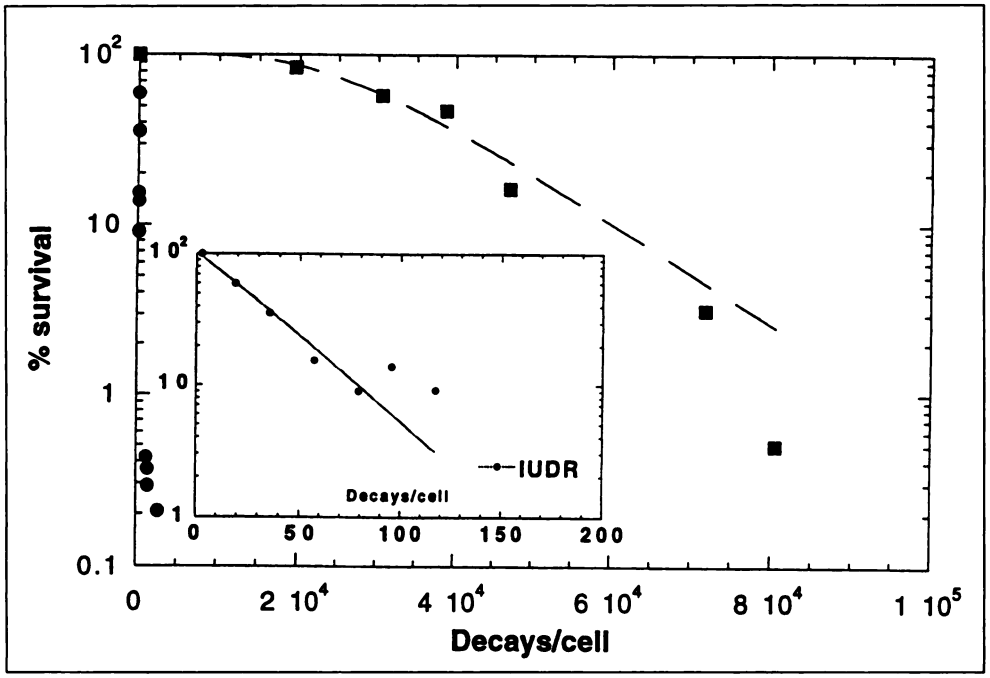

FIGURE 8. Survival curves of HT-1080 cells treated with ^{125}I-ODN (■) and ^{125}I-UdR (●). The data were plotted as a function of accumulated decays per cell calculated from the uptake values (see "Materials and Methods") and fitted as described in the text. For ^{125}I-ODN, D_0 = 14381 ± 2206 (n = 6.89 ± 2.63); for ^{125}I-UdR, D_0 = 32.59 ± 6.20 (n = 1.13 ± 0.28). Due to the very large difference between decays per cell for ODN and I-UdR, the survival curve for ^{125}I-UdR is shown separately in the inset.

^{125}I-UdR and ^{125}I-ODN, the distribution of decays between nucleus and cytoplasm was taken from the autoradiography data (Fig. 3). The ratios were 90% in nucleus, 10% in cytoplasm for ^{125}I-UdR and 75% in nucleus, 25% in cytoplasm for ^{125}I-ODN. ^{125}I-antipyrine was considered to be distributed uniformly through the cells and medium, while ^{125}I-BSA was assumed to be outside the cells. For dose calculation, the average volumes of the nuclei and the HT-1080 cells were estimated as 500 and 4000 fl, respectively, and the concentrations of the cells were taken as 5×10^6/ml. The D_{37} nuclear doses calculated, based on the above assumptions, are 36 Gy for ^{125}I-ODN, 0.1 Gy for ^{125}I-UdR, 1.5 Gy for ^{125}I-antipyrine and 0.32×10^{-6} Gy for ^{125}I-BSA. Obviously, the total dose delivered to the nucleus does not correlate with the radiotoxicity of the ^{125}I-labeled compounds.

Our data demonstrate that the radiotoxicity of Auger electron emitters is determined by the dose delivered to nuclear DNA, not to the nucleus. In other words, the nuclear localization alone does not ensure high radiotoxicity. A clear distinction must be made among DNA-incorporated, DNA-bound, DNA/chromatin-localized and nucleus-localized Auger electron emitter-labeled compounds. The importance of the subnuclear localization of ^{125}I-labeled compounds for interpretation of survival data was noticed previously in the studies of ^{125}I-labeled DNA intercalators (*11,12,35*).

Our data are also important for the development of ODN-based radiotherapeutics (*8,9*). We have previously proposed an approach for targeting genomic rearrangements that often occur in cancer cells with ODN labeled with Auger electron emitters. We have shown that decay of ^{125}I incorporated into a triplex-forming oligonucleotide produces DSB in the target duplex on triplex formation in vitro (*8*). The breaks occur with an efficiency close to one break per decay (*8,9*). These findings make ODN very promising vehicles for gene radiotherapy via sequence-specific breakage of genomic DNA inside the cells.

TABLE 1
D_{37} Values for Iodine-125 Decays in Labeled and Frozen HT-1080 Cells

Agent	D_{37} (decays/cell)	D_{37} (decays/μm^3)
^{125}I-ODN	1.3×10^4	0.24
^{125}I-antipyrine		0.49
^{125}I-BSA		1.92
^{125}I-UdR	30	

To attain D_{37}, decays were accumulated: for ^{125}I-ODN, ~13 days; for ^{125}I-antipyrine, ~12 days; for ^{125}I-BSA, ~17 days; and for ^{125}IUdR, ~7 days.

CONCLUSION

To study the radiotoxic effects of the Auger electron emitter ^{125}I delivered to the nucleus by ODN, we investigated the distribution, stability and radiotoxicity of ^{125}I-ODN in human cells. We conclude that despite the preferable nuclear localization, the bulk of ^{125}I-ODN are not closely associated with genomic DNA. Our data demonstrate that the radiotoxicity of Auger electron emitters is determined by the dose delivered to nuclear DNA, not to the nucleus. We showed that relatively high intracellular concentrations of ^{125}I-ODN can be achieved without causing significant cell death. Therefore, decay of ^{125}I in unbound ODN will not damage cells. This is a very promising result for our proposed trial of ^{125}I-ODN as sequence-specific DNA cleavage agents for gene radiotherapy. It is also important for developing other vehicles to deliver ^{125}I to genomic DNA.

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5-Fluoro-1-(2′-Deoxy-2′-Fluoro-β-D-Ribofuranosyl) Uracil Trapping in Morris Hepatoma Cells Expressing the Herpes Simplex Virus Thymidine Kinase Gene

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The planning and individualization of gene therapy with suicide genes such as herpes simplex virus thymidine kinase (HSV-tk) necessitates the assessment of the enzyme activity expressed in the tumor. This can be done by uptake measurements of specific substrates for HSV-tk. Due to the molecular structure of 5-fluoro-1-(2′-deoxy-fluoro-β-D-ribofuranosyl)uracil (FFUdR), it may be a substrate for both the mammalian thymidine kinase and HSV-tk. **Methods:** Using a HSV-tk-expressing rat hepatoma cell line and a control cell line (bearing the empty vector) the uptake of ^{3}H-FFUdR was determined with increasing incubation periods. Furthermore, measurements with graded mixtures of HSV-tk-expressing cells and control cells were made. To elucidate the mechanism of FFUdR transport into cells, a series of inhibition/competition experiments was performed with challenge inhibitors of the nucleoside and the nucleobase transport systems. **Results:** The uptake studies with tritiated FFUdR revealed a 14- to 19-fold higher accumulation in the HSV-tk-expressing cell line compared to the control cell line. While the ^{3}H-FFUdR uptake was 3- to 4-fold higher than the ^{3}H-ganciclovir uptake in the HSV-tk-expressing cells, it was also higher in control cells (5-fold). Furthermore, FFUdR accumulation was linearly correlated with the amount of HSV-tk-expressing cells. FFUdR uptake and growth inhibition by therapeutic doses of ganciclovir were highly correlated, with $r = 0.96$. Inhibition/competition experiments showed that FFUdR is transported mainly by the equilibrative and the concentrative nucleoside transporter but not by the nucleobase transport systems. **Conclusion:** The FFUdR uptake is an indicator of the HSV-tk activity in tumor cells and can be used as a prognostic marker during gene therapy with HSV-tk. The relative merits of ganciclovir and FFUdR as specific substrates for HSV-tk will need to be further explored in vivo.

Key Words: gene therapy; specific substrate; hepatoma; PET

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