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Effect of 5-[¹²⁵I]iodo-2'-deoxyuridine uptake on the proliferation and pluripotency of human embryonic stem cells

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Abstract

Purpose—Human embryonic stem cells (hESC) hold a great potential for regenerative medicine because, in principle, they can differentiate into any cell type found in human body. In addition, studying the effect of ionizing radiation (IR) on hESC may provide valuable information about the response of human cells to IR exposure in their most naive state, as well as the consequences of IR exposure on the development of organisms. However the effect of IR, in particular radionuclide uptake, on the pluripotency, proliferation and survival of hESC has not been extensively studied.

Methods—In this study we treated cultured hESC with 5-[¹²⁵I]iodo-2'-deoxyuridine (¹²⁵IdU), a precursor of DNA synthesis. Then we measured the expansion of colonies and expression of pluripotency markers in hESC.

Results—We found that uptake of ¹²⁵IdU was similar in both hESC and HT1080 human fibrosarcoma cells. However, treatment with 0.1 uCi/ml ¹²⁵IdU for 24 hours resulted in complete death of the hESC population; whereas HT1080 cancer cells continued to grow. Treatment with a tenfold lower dose ¹²⁵IdU (0.01 uCi/ml) resulted in colonies of hESC becoming less defined with numerous cells growing in monolayer outside of the colonies showing signs of differentiation. Then we analyzed the expression of pluripotency markers (octamer-binding transcription factor 4 (Oct-4) and stage-specific embryonic antigen-4 (SSEA4)) in the surviving hESC. We found that hESC in the surviving colonies expressed pluripotency markers at levels comparable with those in the non-treated controls.

Conclusions—Our results provide important initial insights into the sensitivity of hESC to IR, and especially that produced by the decay of an internalized radionuclide.

Keywords

Auger emitters; stem cells; cell biology

Introduction

Pluripotent human embryonic stem cells (hESC) are able to differentiate into various cell types found in the human body; and thus carry great potential for regenerative cell replacement-based medicine. The full realization of the therapeutic potential of hESC in practice may require labeling these cells with radionuclides for *in vivo* analysis of their fate that can subsequently be tracked with imaging modalities such as positron emission

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Declaration of interest

Authors declare no conflicts of interest.

tomography (PET) and X-ray computed tomography (CT) that expose hESC to appreciable doses of ionizing radiation (IR).

The majority of our knowledge about the molecular mechanisms of cellular response to IR exposure is based on experiments with traditional cell culture models. Of these models, cancer cell lines are used most often, which may seem quite reasonable given that radiation therapy of cancers is the main clinical application of IR in biomedicine. However, it is quite obvious that normal cell lines and cancer cell lines have different responses to IR. Various primary cell lines obtained from different tissues, such as primary fibroblasts, are also often used in radiobiological studies. Results obtained from cultures of such terminally differentiated cells may not reflect the response to IR of a normal tissue that consists of various cell types including progenitor and tissue stem cells.

In the past decade there has been great progress in developing and maintaining in culture pluripotent embryonic stem cells (ESC). Moreover, methods have been developed to differentiate these cells into various cell lineages and, in many cases, into fully differentiated cell lines sometimes mimicking function of the corresponding cells in tissues. These developments open a new horizon for cellular radiobiology. Researchers can now use ESC to study the effects of IR on the survival, proliferation maintenance of pluripotency, and differentiation of these cells. Several studies using external sources of radiation have been reported recently (Wilson et al., 2010, Momcilovic et al., 2009, Momcilovic et al., 2010, Sokolov and Neumann, 2010, Sokolov et al., 2011) showing that hESC are hypersensitive to IR. For example, in our previous study, we have showed that exposure of hESC to low doses of IR initially caused massive apoptosis, however, did not interfere with the ability of the surviving fraction to maintain pluripotency (Sokolov et al., 2010). However, the effect of radioisotope uptake on ESC remains largely unknown.

Radiobiological effects produced by internalized radioisotopes can be quite different from those produced by external sources of IR. The same radiation dose delivered by a radioisotope will have a different temporal and spatial distribution from that delivered by a relatively short pulse of uniformly distributed gamma- or x-rays. This is especially true for the Auger electron emitting radionuclides that deposit most of their energy within nanometers from the decay site (for review see Kassis, 2004).

Herein, we investigated the effect of decay of an Auger electron emitter ^{125}I incorporated into the DNA of cultured hESC during their replication as a precursor of DNA synthesis ^{125}IdU . Previously, treatment with ^{125}IdU was shown to be highly radiotoxic for cultures of cancer cell lines (Yasui et al., 1985, Makrigiorgos et al., 1989, Schneiderman et al., 1991, Sedelnikova et al., 1998, Walicka et al., 2001). Our measurements of the rate of uptake of ^{125}IdU show that it was similar to that of cancer cells. However, the same number of ^{125}IdU decays per cell that caused significant cells death in the hESC population did not immediately harm the similarly treated cancer cells. We also immunostained hESC for a surface antigen SSEA4 and a transcription factor Oct4 and show that hESC that survived after ^{125}IdU treatment express these standard markers of pluripotency (Sokolov et al., 2010).

Material and Methods

H1 and H9 hESC lines (WiCell Research Institute, Madison, WI, USA) were maintained in feeder-independent cell culture environments for human embryonic stem cells on BD Matrigel hESC-qualified Matrix (BD Biosciences, San Jose, CA, USA) coated BD Falcon™ qualified surfaces plates in serum-free defined mTeSR1 media (Stem Cell Technology Inc, Vancouver, BC, Canada) at 37°C and 5% CO₂. Growing cells (passage 36-39) were passaged using collagenase IV. Media was changed every day. H9 and H1 hESC maintained

for 8 days in culture were passaged into experimental plates, and grew for three days for colonies to form before treatment with ^{125}IdU . HT1080 human fibrosarcoma epithelial cell line (ATCC, Manassas, VA, USA) was maintained as was described (Sedelnikova et al., 1998).

Cells were treated with ^{125}IdU (Perkin Elmer, Waltham, MA, USA, 2200 Ci/mmol, 1 mCi/ml) in 60×15 mm cell culture dishes (BD Biosciences) at concentrations indicated in the results section. Cells were incubated with ^{125}IdU for 24 hours (a doubling time for these cells) to insure that all cells took up the radionuclide. Then cells were washed twice with fresh media to remove unincorporated ^{125}IdU . At each time point indicated in the results section cells were collected by trypsinization for cell count and ^{125}I uptake measurement. Cells were counted using a hemocytometer (twice) and their radioactivity was measured with an MPD γ -counter (Biotraces, Inc, Herndon, VA, USA). The average number of decays per minute (dpm) per cell was calculated by dividing measured dpm in the number of cells in the sample.

To count the number of cells within a colony, hESC were stained with 1.25 $\mu\text{g/ml}$ Hoechst 33342 (Invitrogen, Carlsbad, CA, USA) in phosphate-buffered saline (PBS) solution. After 90 min the Hoechst solution was aspirated and new culture medium was added. Stained colonies were analyzed using Zeiss Axiovert 200 microscope (Carl Zeiss, Thornwood, NY, USA) and cell counts were performed for 30 colonies of different sizes. For further experiments we used colony area as a measure of their proliferation. hESC were grown on gridded plates to help locate colonies. At various time points, phase-contrast pictures of individual colonies were taken and the area of the colonies was measured with AxioVision Software (Carl Zeiss) by manually outlining the borders of a colony.

For immunohistochemistry, cells were grown on glass-bottom LabTek® two-well Chamber Slides™ (BD Biosciences) in the feeder-free conditions described above. The cell cultures were fixed with 4% paraformaldehyde for 10 minutes, and then permeabilized with 0.1% Triton-X-100 in PBS for 5 minutes, and stained with primary antibodies by incubation for 1-2 hours and the appropriately coupled Alexa Fluor secondary antibodies (Invitrogen) for 1 hour. All secondary antibodies were tested for nonspecific immunoreactivity. The following primary antibodies were used: Oct-4, SSEA4, (Santa Cruz Biotechnology, Santa Cruz, CA, USA), After mounting in antifade media (VectaShield, Vector Laboratories, Inc., Burlingame, CA, USA), the samples were examined using an Axioplan Zeiss epifluorescent microscope. The digital camera exposure time and microscope settings were kept constant across all corresponding samples.

Results

We first treated hESC with 0.1 $\mu\text{Ci/ml}$ of ^{125}IdU based on concentrations used in previous studies with cancer cells lines (Yasui et al., 1985, Makrigiorgos et al., 1989, Schneiderman et al., 1991, Walicka et al., 2001). At this concentration of ^{125}IdU the majority of hESC became detached after 24 hours of incubation; therefore, all the following experiments were carried out at lower concentrations of ^{125}IdU , i.e. 0.01 $\mu\text{Ci/ml}$ and 0.05 $\mu\text{Ci/ml}$. Figures 1A and 1B shows typical colonies of H1 and H9 hESC treated with 0 $\mu\text{Ci/ml}$ (control), 0.01 $\mu\text{Ci/ml}$ and 0.05 $\mu\text{Ci/ml}$ of ^{125}IdU 24, 48 and 96 hours after treatment. In the mock treated samples (top row) the colonies grew in size after 48 hours and even became overgrown after 96 hours resulted in appearance detached floating cells, especially in the H1 hESC sample (Figure 1A). Treatment with 0.01 $\mu\text{Ci/ml}$ of ^{125}IdU (middle row) resulted in significant cell death after 96 hours and appearance of colonies with holes in the middle for both hESC lines. Treatment of H1 cells with 0.05 $\mu\text{Ci/ml}$ of ^{125}IdU (bottom row) leads to massive cell death after 48 hours. Only a few cells survived in tiny colonies after 96 hours. Almost all H9

cells were dead 48 hours after treatment with 0.05 $\mu\text{Ci/ml}$. Treatment of HT1080 cells with the same concentrations of ^{125}IdU did not result in any visible cell death, or slow their proliferation (data not shown).

Cell count and uptake of ^{125}IdU in terms of decay per minute per cell 24 and 48 hours after treatment are shown in Table 1. After 0.01 $\mu\text{Ci/ml}$ treatment ^{125}IdU uptake was very similar for all types of cells; however, the number of attached HT1080 cells almost doubled between 24 and 48 hours time points while the number of both H1 and H9 hESC significantly declined. Lower uptake of ^{125}IdU by H1 and H9 cells after 0.05 $\mu\text{Ci/ml}$ treatment can be explained by significant cell death even 24 hours after treatment. We believe that the cells that took up more ^{125}IdU by being at the early S-phase at the time of treatment died first while cells that took up less ^{125}IdU remained attached at this time point. After 0.05 $\mu\text{Ci/ml}$ treatment, the number of HT1080 cells almost doubled while numbers of H1 and, especially, H9 hESC drastically declined. Therefore, we concluded that hESC take up ^{125}IdU as effectively as cancer HT1080 cells, but they are significantly more sensitive to ^{125}IdU than HT1080 and H9 are more sensitive than H1 cells.

We then measured the ^{125}I content in hESC 24, 48 and 96 hours after treatment with ^{125}IdU . Data presented in Figure 2 show that the number of dpm per cell significantly decreased within the cells that remained attached with increasing time after treatment (note that y-axis scale is logarithmic). This happened along with decreases in the total number of attached cells (Table 1). Therefore, the cells surviving longer after ^{125}IdU treatment contain considerably less ^{125}I than those detached and presumably dead cells.

Next, we measured the effect of ^{125}IdU treatment on proliferation of hESC. The classical assay to evaluate the sensitivity of cultured cells to radiation is clonogenic survival (Elkind et al., 1967). However, this assay is not applicable to hESC because if their colonies are broken down to single cells their plating efficiency is very low, less than 1% (Chan et al., 2008). An alternate way of assessing radiosensitivity is to measure the growth curve of the cultured cells. This is usually done by plating equal amounts of cells into several dishes, treating them with IR or radionuclide and counting cells in the dishes after certain periods of time. Because hESC grow and propagate in colonies, it is almost impossible to seed equal amounts of them into several plates or dishes. One way to work around this problem would be to measure the total area of colonies of hESC and use it as a measure of the total cell number. This approach is based on an assumption that the area of a colony is proportional to the number of cells in it. To test this hypothesis, we directly counted the number of cells in several colonies of different sizes, and compared those numbers with the areas of colonies. We limited our count to the colonies that grew as monolayer with no visible “piling up” of cells in multiple layers. The results presented in Figure 3 show that there is no obvious correlation between the size of a colony and the density of the cells in it. On the contrary, density of the cells varies considerably between individual colonies, and, therefore, total area of the colonies on the plate may not be directly proportional to the total number of the cells. In addition, different colonies may have different response to ^{125}IdU treatment. Therefore, instead of measuring the total area of colonies on a plate we measured the effect of ^{125}IdU treatment on the areas of individual colonies.

Figure 4 shows results of such measurements for several colonies of H9 hESC treated with 0.01 $\mu\text{Ci/ml}$ or non-treated (control) with ^{125}IdU for several days after the treatment. The area of the colonies of non-treated cell (Figure 4A) increased exponentially with time, reflecting an exponential increase in the number of cells in colonies due to cell division. The area of the colonies of hESC treated with 0.01 $\mu\text{Ci/ml}$ of ^{125}IdU (Figure 4B) continues to increase after addition of ^{125}IdU at day 0, but then rapidly declines on day 3 in agreement with the pictures shown in Figure 1. The shapes of the growth curves are different for

different colonies; larger colonies, like colony #1 (Figure 4B), expand faster than smaller ones (colony #3). However, they all diminished to the almost the same smaller size on day 3 after ^{125}IdU treatment. After this, some colonies recover and continued to grow exponentially while we were observing them for several more days. Some colonies, like colony #2, never recovered and even completely disappear after day the 3 time point. Similar results were obtained for H1 cells (data not shown).

The results shown in Figure 4 demonstrate that by measuring the area of the colonies we could quantify the proliferation of hESC. They also prove that all the colonies lose cells on day 3 after ^{125}IdU treatment. The bigger the colony the more cells it loses, but most recover and continue to grow exponentially. We followed the recovered colonies for several days, up to day 7 (Figure 5). They looked like normal healthy hESC colonies except that in some cases we observed loosely packed cells growing next to a colony (Figure 5, top row, middle panel). We then checked for the presence of pluripotency markers in the recovered colonies.

We tested the surviving and recovered colonies for the expression of two well-established pluripotency markers; a surface antigen SSEA4 and a transcription factor Oct-4. Figure 6 shows staining with SSEA4 and Oct-4 antibodies of the colonies of H1 cells on day 7 after ^{125}IdU treatment. They were positive for both SSEA4 and Oct-4 antibody staining. The intensity of the staining was similar in the control and ^{125}IdU treated colonies. However, the loose cells that grow next to the colony were negative for the antibody staining; therefore we concluded that they were differentiated. Similar results were obtained with H9 hESC line (data not shown). Therefore, we believe that hESC that survived ^{125}IdU treatment fully recovered and remained pluripotent, however, further and more strict tests like teratoma formation assays are required to ultimately prove this point.

Discussion

Decay of Auger electron emitting radioisotopes, in particular ^{125}I , incorporated into genomic DNA was found extremely radiotoxic for cultured cells with the relative biological effectiveness ranging from 7 to 10 in different studies (Sastri, 1992, Yasui, 1992). Most of these measurements were made with a clonogenic survival assay that is widely used in radiobiology (Yasui et al., 1985, Makrigiorgos et al., 1989, Schneiderman et al., 1991, Yasui, 1992, Walicka et al., 2001). However, interpretation of clonogenic survival data may be different for cells exposed to an external source of radiation than for cells treated with internalized radionuclide. In the first case the action of IR is finished as soon as the cells are removed from an irradiator, while in the second case cells can be exposed to decays of non-cleared radionuclide even during the colony formation assay. This is especially true for treatments with ^{125}IdU . Because of its structural resemblance to thymidine ^{125}IdU is practically not detected by cellular DNA repair systems and therefore once incorporated into DNA, can remain there for several generations (Mitchell et al., 1989). In addition ^{125}I has a long (60 days) half-life.

To study the effect of ^{125}IdU treatment on hESC we used an alternative to the clonogenic assay approach. Clonogenic survival studies are not applicable to cultures of hESC because they grow in colonies and if separated to single cells have extremely low plating efficiency (Chan et al., 2008). We first show that the rate of ^{125}IdU uptake is similar for hESC and cancer HT1080 cells and then we measured growth curves of these cells. In the case of hESC we took advantage of their growth in colonies, and as a measure of their proliferation we used the area of the colonies. We found that treatment with moderate amounts (0.01 and 0.05 $\mu\text{Ci/ml}$) of ^{125}IdU did not have a noticeable effect of the growth of HT1080 cells during first two days after the treatment. In contrast, treatment of hESC with the same dose and resulted in similar uptake of ^{125}IdU caused massive cell death. Nevertheless, the

colonies that survived and recovered remained positive for pluripotency markers and virtually free from ^{125}IdU .

There are at least two explanations for the above observations; some cells did not take up ^{125}IdU during the treatment (as it was suggested for surviving cancer cells), or hESC have a mechanism to detect and remove ^{125}IdU from their DNA. Further studies are required to establish which of these two is utilized by colonies of hESC to survive and to continue self-renewal and proliferation. Our results demonstrate that hESC initially respond to DNA damage by massive cell death, presumably by apoptosis as was the case after gamma irradiation in our previous studies (Sokolov et al., 2011, Sokolov et al., 2010). Such a response may be an important protective feature of stem cells because accumulation of DNA damage-causing mutations in hESC will be passed on to all cells in a developing organism. At the same time, colonies manage to preserve those cells that presumably retain an undamaged genome, or an effectively repaired genome, that were able to expand to pluripotent colonies thus showing resiliency of the hESC colonies. On the other hand, appearance of apparently differentiated cells (Figure 5) may indicate on their selective expansion due to the advantage of being more radioresistant than pluripotent hESC. Obviously further and more extensive studies are required to understand the mechanism of hESC survival after ^{125}IdU treatment. However, it is now clear that hESC react differently to radiotoxic insults than differentiated cells, and represent an important model to study effect of radiation on developing organisms.

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Figure 1.

Colonies of H1 (A) and H9 (B) hESC non-treated (control) and treated with 0.01 and 0.05 $\mu\text{Ci/ml}$ of ^{125}IdU , 24, 48 and 96 hours after treatment. Phase-contrast images of live colonies were taken with Axiovert 200 microscope at 10 $\times$ magnification. A 100 μm bar is shown on the bottom right panel.

Figure 2. Radioiodine content expressed in dpm/cell in H1 and H9 hESC 24, 48, and 96 hours after treatment with $^{125}\text{IIdU}$. Only cells that remained attached to the matrigel were counted. Cells were counted twice; average of the two measurements and standard error are shown.

Figure 3. Cell density in colonies of H9 hESC. A. Hoechst 33342 stained colony with shown area of measurement and manual cell count. A fluorescent image was taken with Axiovert 200 microscope at 10 $\times$ magnification. A 100 μm bar is shown. B. Cell density in number of cell per mm^2 plotted against area for 30 different colonies.

Figure 4.

Growth curves for colonies of H9 hESC. Area of several colonies of non-treated (A) and treated with 0.01 $\mu\text{Ci/ml}$ of ^{125}IdU (B) cells on days 0 to 5. Numbers on panel B identify colonies that are discussed in the text. Area of each colony was measured three times; average values are shown. Standard errors of these measurements were less than 3%, they are not shown.

Figure 5.

Colonies of H1 and H9 hESC non-treated (control) and treated with 0.01 and 0.05 $\mu\text{Ci/ml}$ of ^{125}IdU , on day 7 after treatment. Phase-contrast images of live colonies were taken with Axiovert 200 microscope at 10 $\times$ magnification. Phase-contrast images of live colonies were taken with Axiovert 200 microscope at 10 $\times$ magnification. A 100 μm bar is shown on the bottom right panel.

Figure 6.

Expression of pluripotency markers. Microphotographs showing colonies of H1 hESC non-treated (control) and treated with 0.01 and 0.05 $\mu\text{Ci/ml}$ of ^{125}IdU , on day 7 after treatment. Cells were fixed and incubated with antibody against SSEA4 and Oct-4 and then stained with Alexa Fluor secondary antibodies. Fluorescent images of fixed colonies were taken with Axiovert 200 microscope at 10 $\times$ and 20 $\times$ (top left and bottom right panels) magnification.

Table 1

Number of cells (in millions) and dpm/cell 24 and 48 hours after treatment with 0.01 and 0.05 $\mu\text{Ci/ml}$ of ^{125}IdU

	0.01 $\mu\text{Ci/ml}$			0.05 $\mu\text{Ci/ml}$		
	24 h		48h	24 h		48 h
	Cells	Dpm/cell	Cells	Cells	Dpm/cell	Cells
HT1080	2.66	0.03	5.22	1.27	0.22	2.37
H1	2.1	0.02	0.69	0.74	0.15	0.49
H9	1.11	0.03	0.15	1.32	0.10	0.13