Production of DNA Double Strand Breaks in Human Cells due to Acute Exposure to Tritiated Water (HTO)

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INTRODUCTION

The average and maximum energies of the beta emission from \textsuperscript{3}H are 5.69 keV and 18.6 keV respectively. The average range in water (or soft tissues), around 0.5 \(\mu\)m (500 nm), is considerably less than the typical diameter of a cell (10-30 \(\mu\)m), and even of a cell nucleus (5-10 \(\mu\)m), thus the micro-location of the tritium atom may well be crucial in determining its biochemical consequences. Due to the high ionization density of the beta particles emitted by tritium (about 400 ion pairs/\(\mu\)m) possible interaction of tritium beta radiation with DNA may play a significant role.

Tritiated water (HTO) is the main chemical form in which tritium is found in the environment. In the body it may be retained as organically bound tritium (OBT), binding to biological molecules or remaining as OBT with various degrees of solubility. OBT can be retained in the human body much longer than HTO and therefore the dose arising from OBT can reach 50\% of the total tritium dose \textsuperscript{(1)}.

Histones are major protein components of chromatin. They function as spools around which DNA winds and play an important role in the regulation of gene expression. In the absence of histones, the DNA in chromosomes would be unmanageably long, as human cells each have about 1.8 m of DNA. During mitosis, DNA is duplicated and condensed, resulting in about 120 \(\mu\)m of chromosomes \textsuperscript{(2)}. It was recently reported that the phosphorylation of histone H2AX on serine residue 139 (\(\gamma\)-H2AX) is associated with Double Strand Breaks (DSB) sites in DNA \textsuperscript{(3)}, which indicates the possibility of research based on the detection of DSBs in DNA. The phosphorylated megabase chromatin domain surrounding the DSB can be immunostained and visualized as discrete foci by fluorescence microscopy \textsuperscript{(4)}, as each DNA DSB formed produces a visible \(\gamma\)-H2AX focus \textsuperscript{(5)}. Since 1 Gy of radiation produces approximately 60 DSBs/cell, doses of a few mGy should be distinguishable from the background, and it was recently shown that the exposure to 1 mGy of X-rays induces a significant increase in H2AX phosphorylation in primary human fibroblasts \textsuperscript{(6)}. Thus, immunostaining of \(\gamma\)-H2AX may represent a sensitive biomarker of exposure \textsuperscript{(7)}.

The scientific literature contains practically no information about the effect of acute exposure from HTO on the integrity of the DNA, and the present work presents some preliminary results on this subject. The investigation on the formation of the Double Strand Breaks was done by the detection of the phosphorylated histone, \(\gamma\)H2AX. For this research we used Human malignant osteoblast MG-63 cells, and normal peripheral blood lymphocytes.

EVALUATION OF THE EXPOSURE DOSES IN HTO MEDIA

For investigation of the DSBs effects in DNA, cells were exposed to various activities in tritium liquid solutions. A tritium concentration of 13,220 Bq/ml in solution was defined as 1 Ac. The exposure effect was tested using solutions from 0.5 Ac to 16 Ac. Evaluation of the tritium dose in the HTO medium was done based on the basic definition of energy absorbed per mass unit (Grey units).
The average energy of a beta particle emitted from tritium is 5.7 keV. Assuming that all beta particles are absorbed in the aqueous volume (due to their short range), the energy absorbed in 1 kg of a 16 Ac solution is:

\[ 2.115 \times 10^5 \text{[Bq/ml]} \times 5.7 \text{[keV]} \times 1.6 \times 10^{-16} \text{[joule/keV]} \times 10^3 \text{[ml/kg]} = 1.93 \times 10^{-7} \text{[joule/kg/sec]} \]

1 Gy is defined as 1 [joule/kg]; therefore, the dose rate corresponding to a tritium activity of 16 Ac is 1.93 \times 10^{-7} \text{[Gy/sec]}. For an incubation time of 3 h, the total dose will be:

\[ 1.93 \times 10^{-7} \text{[Gy/sec]} \times 1.08 \times 10^4 \text{[sec]} \times 10^3 \text{[mGy/Gy]} = 2.09 \text{[mGy]} \]

Scarpa et al. \(^8\) also evaluated the absorbed dose for a \(\beta\)-emitting isotope uniformly distributed in a homogeneous medium. Applying the Equation used by Scarpa to the tritium solution described above gives a dose value of 1.51 mGy, which is close to that evaluated above, based on the most basic definitions.

The exposure of 2.09 mGy corresponds to a solution containing a tritium activity of 16 Ac (211,533 Bq/ml) and an exposure time of 3 hours. Thus the basic exposure factor is 0.0435 Bq·Ac^{-1}·hr^{-1}. Based on this factor, the exposure for each tritium concentration and exposure time can be calculated. The doses used in this study ranged from 0.065 mGy to 16.72 mGy, which were obtained with HTO activities in the range 6.61 \times 10^3 \text{Bq/ml} to 2.11 \times 10^5 \text{Bq/ml (0.5 - 16 Ac)}. These doses are classified in the literature as low doses \(^9\), \(^10\), \(^11\).

**RESULTS**

**Detection by Immunofluorescence**

One group of cells was incubated for 3 h and for 24 h in media containing different HTO concentrations. The second group was exposed for the same total incubation time to 1 \(\mu\text{M}\) etoposide (known to cause DSBs). At the end of incubation, the cells were detached from the plastic flasks by trypsinization, rinsed twice with large volumes of PBS, diluted to the desired concentration, and dispersed onto glass slides. After performing a micro assay using an incubation step of monoclonal antibody against \(\gamma\text{H2AX}\) (Biolegend\textsuperscript{TM}, cat#: 613402), they were diluted 1:100 and an additional incubation step with DAPI (a minor groove dye of DNA that is used for staining nuclei) was performed. The cells were then analyzed by fluorescence microscopy for cy-3 and DAPI emissions.

The immunofluorescence results (Figures 1-4) for different tritium exposures show that DSBs occurred in DNA only after the exposure of the cells to 1 \(\mu\text{M}\) etoposide for 24 h and after to 16 Ac HTO for 24 h (Figures 2 and 3). Typical fluorescent foci were only observed after exposure to 16 Ac activity in the DAPI nuclear staining area (~10% of total cells exhibited fluorescent foci).
Detection by Western Blot

The western blot analytical technique was used to detect the formation of the phosphorylated protein (serine-139), γ-γH2AX. To lyse the cells, the different cellular mixtures (of cells exposed to HTO for 3 h) were treated with RIPA buffer. The membranes and the DNA fraction were separated from the cellular extracts, and the DNA fraction was treated once more with RIPA buffer supplemented with SDS to achieve a final concentration of 0.1% (or according to Abcam's procedure). Equivalent amounts of 25 μg (except 12 μg for the Etoposide) of the membranes and DNA fraction from the various treatments were used for the western blot analysis. The proteins that were transferred from the 15% acrylamide gel to the nitrocellulose membrane were reacted overnight with a 5 μg monoclonal anti-γH2AX antibody (BioLegend™, cat#:613402) diluted in 5 ml PBS containing 5% BSA (fraction 5, Sigma) and 0.05% Tween-20. The
nitrocellulose membrane was rinsed three times, 10 min each time, in PBS containing 0.05% Tween-20 and then reacted for 1 h with a secondary antibody, goat anti-mouse #HRP conjugated (Santa Cruz™), diluted 1:10,000 (in the same buffer), after which it was rinsed again in PBS containing 0.05% Tween-20. The immune complexes were detected by an ECL system followed by autoradiographs. Band intensities were quantified by densitometric scans using EZQuant-Gel image processing and analysis software (Rehovot, Israel).

The nitrocellulose membrane was also allowed to react with an anti H3 histone antibody (Abcam#Ab1791) to enable a quality control test of the protein quantities used (Figures 5 and 6).

![Figure 8: γH2AX detection in control cells and in cells exposed to either 120 μM etoposide or HTO for 24 h.](image1)

![Figure 9: H3 histone detection in control cells and in cells exposed to either 120 μM etoposide or HTO for 24 h.](image2)

The similar band areas exhibited by the different treatments indicate that the same quantities of fraction extracts were used (Figure 6). The densitometric analysis of the different samples also indicated that equal amounts of proteins were loaded on the gels. The variability among the samples was in the range of 6%. As the etoposide treatment was an important positive control for H2AX phosphorylation, two protein concentrations were used 25 μg and 12μg.

As seen from the results presented in Figure 5, only the 24-h exposures to 16 Ac tritium and to 120 μM of etoposide caused DSBs to form in DNA (Figure 5).

CONCLUSIONS

It was found that low HTO dose exposure of 16.72 mGy can cause DSBs after exposing MG-63 cells for 24 h. This dose did not cause any DSBs in normal stimulated peripheral blood lymphocytes (data are not shown), probably due to the inherent metabolism and replication differences between the two cell types. The capability of tritium (as HTO) to cause DSBs, proves that this radionuclide has a potential to cause DNA damage.

REFERENCES