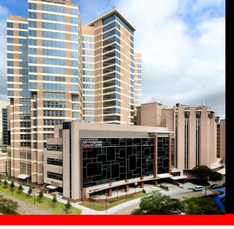


Is proton radiation more effective than photon radiation at inducing senescence?

Emma Moran, Mariam Ben Kacem, David Flint, David Martinus, Mandira Manandhar, Simona F. Shaitelman and Gabriel O. Sawakuchi

Department of Radiation Physics, The University of Texas MD Anderson Cancer Center, Houston, TX

THE UNIVERSITY OF TEXAS MD Anderson Cancer Center

Background

Breast Cancer is …

- the most diagnosed type of cancer for women in the United States[1].
- the 2nd most deadly type of cancer for women in the United States[1].

BRCA = BReast CAncer genes

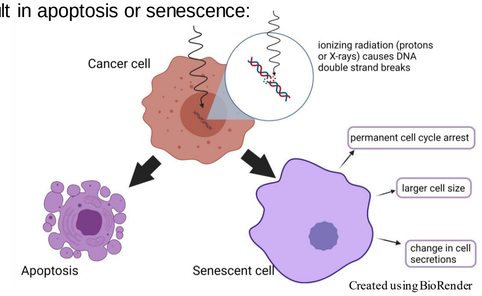
- BRCA1 and BRCA2 encode proteins that repair damaged DNA, preventing cancer from developing – harmful mutations prevent this process and cause cancer[2].
- Everyone inherits 1 copy of BRCA1 and BRCA2 from each parent - harmful BRCA mutations can also be inherited[2].
- We use 2 human breast cancer cell lines: HCC1937 (BRCA-mutated), HCC1937-BRCA (BRCA-restored), and 1 mouse breast cancer cell line: 4T1.

Senescence = a state of permanent cell-cycle arrest[3]

- Replicative senescence – caused by telomere shortening over many replication cycles.
- Stress-induced senescence – triggered by some type of external stress on the cell (such as radiation).

Radiotherapy

- Ionizing radiation – protons or photons – damages DNA and can result in apoptosis or senescence:



Hypothesis

We hypothesized that protons would cause greater senescence than X-rays for the breast cancer cell lines 4T1, HCC1937, and HCC1937-BRCA.

Methods

Senescence-associated β-galactosidase (SA β-gal) is a biomarker of cellular senescence, and we used two methods of SA β-gal detection to identify and quantify senescent cells.

1) Histochemical Assay[4]

- Plate cells in 6-well plates using glass coverslips.
- Irradiate with 10 Gy protons (9.9 keV/µm) and photons (6 MV X-rays) when cells are sub-confluent.
- Allow 7 days for senescence to develop, then stain cells with the Invitrogen β-gal Staining Kit, which uses:
  - X-gal = substrate of SA β-gal that releases an insoluble blue compound when cleaved. Senescent cells detected by presence of blue spots.

Methods (continued)

2) Flow Cytometry[4]

- Plate cells in T12.5 flasks.
- Irradiate with 5 Gy protons (9.9 keV/µm) and photons (6 MV X-rays) or treat with H2O2 (200-400 µM) for 1-2h at 37ºC when cells are sub-confluent.
- Allow 7 days for senescence to develop, then prepare cells for flow cytometry:
  - 1 hour incubation with bafilomycin A1 to get rid of background senescence (replicative senescence)
  - 2 hour incubation with C_{12}FDG (5-Dodecanoylaminofluorescein Di-β-D-Galactopyranoside), a substrate of SA β-gal that fluoresces when cleaved
- Run the samples in the flow cytometer

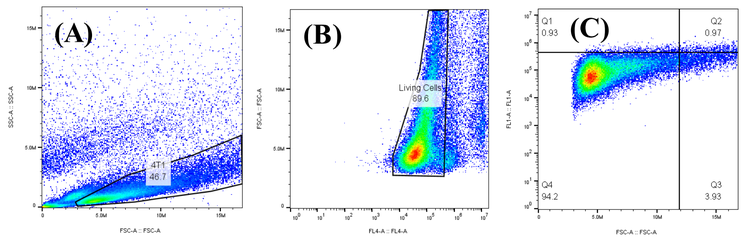


Figure 1. Cytometry plots for non-irradiated 4T1 cells. Bounds on graph (A) isolate 4T1 cells (remove debris). Bounds on graph (B) select only living 4T1 cells. In graph (C), the top 2 quadrants contain senescent cells. For the non-irradiated sample, senescence is set to 2%, and irradiated samples are analyzed relative to the non-irradiated sample. Data represent one independent experiment.

Results: Flow Cytometry

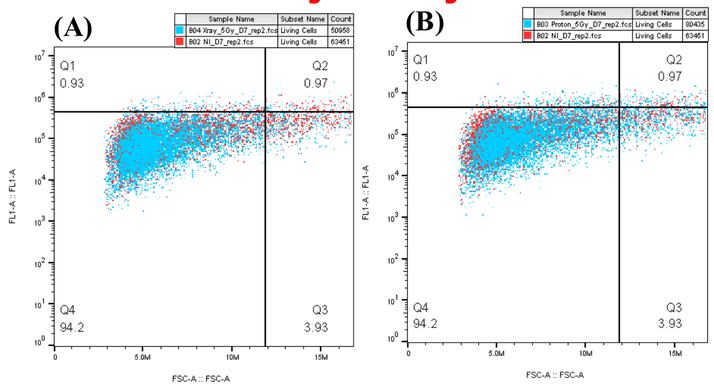


Figure 2. Cytometry plots of 4T1 non-irradiated samples (red) overlaid with 5 Gy X-rays (A), and 5 Gy protons (B) in blue. Each bi-parametric representation (Size (FSC)/C_{12}FDG (FL-1)) represents one independent experiment for at least 5x10^5 living cells.

Results (continued)

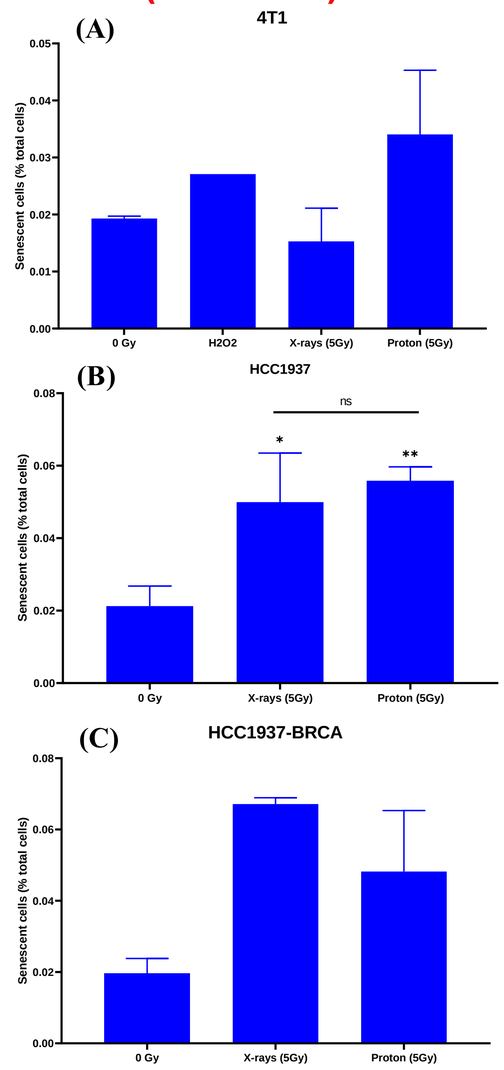


Figure 3. The graphs above show the percent of senescent cells for 4T1 (A), HCC1937 (B), and HCC1937-BRCA (C) at varying radiation types and doses. Each bar represents the mean of independent experiments (2 replicates for 4T1 and HCC1937-BRCA and 4 for HCC1937). Statistical analyses were performed on HCC1937 only (one way Anova: * p<0.05: ** p<0.001 compared to 0 Gy).

Results: X-gal Staining

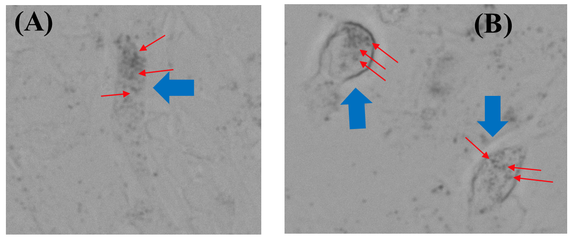


Figure 4. Images obtained from X-gal staining. The small dark spots clustered within cells are the insoluble clue compounds released by X-gal (shown by the red arrows), and the cells that they are clustered within are senescent cells (shown with blue arrows). Images are of 4T1 irradiated with 0 Gy (A) and 10 Gy (B).

Discussion

We are still working on optimizing the X-gal staining protocol. The current images are inconsistent and difficult to highlight differences by microscopy. We are trying to obtain clearer images by using glass coverslips and decreasing the seeding number.

Further replicates of these experiments are currently being conducted. Based on the current cytometry results, it appears that protons are more effective at inducing senescence for 4T1 but X-rays are more effective at inducing senescence for HCC1937-BRCA. We have significantly more senescent cells for HCC1937 after radiation compared to 0 Gy but no differences after proton and X-rays groups.

We expected protons would induce greater senescence because they are more massive than photons and have a higher linear energy transfer (LET), resulting in more double strand breaks and thus greater replicative stress.

After completing our data sets in vitro, we plan to design experiments to evaluate our results in vivo. We hope that our in vitro results are also reflected in vivo and that greater senescence results in better tumor control.

References

(1) Breastcancer.org. (2021, February 4). *U.S. breast cancer statistics*. Breastcancer.org.https://www.breastcancer.org/symptoms/understand_bc/statistics.

(2) National Cancer Institute at the National Institute of Health. (n.d.). *BRCA gene Mutations: Cancer risk and genetic testing fact sheet*. National Cancer Institute. https://www.cancer.gov/about-cancer/causes-prevention/genetics/brca-fact-sheet.

(3) Campisi, J., & d'Adda di Fagagna, F. (n.d.). *Cellular senescence: When bad things happen to good cells*. Nature News. https://www.nature.com/articles/nrm2233.

(4) Debacq-Chainiaux, F., Erusalimsky, J. D., Campisi, J., & Toussaint, O. (2009). *Protocols to detect senescence-associated beta-galactosidase (sa-βgal) activity, a biomarker of senescent cells in culture and in vivo*. Nature Protocols, 4(12), 1798–1806. https://doi.org/10.1038/nprot.2009.191

Acknowledgements: This presentation is supported by the National Cancer Institute through the U54 CA096297/CA096300: UPR/MDACC Partnership for Excellence in Cancer Research Training Program. For further information, please contact Emma Moran at emoran1@mdanderson.org