The use of organoids in the study of radiation response and therapeutic window
Nagle, Peter

IMPORTANT NOTE: You are advised to consult the publisher's version (publisher's PDF) if you wish to cite from it. Please check the document version below.

Document Version
Publisher's PDF, also known as Version of record

Publication date:
2018

Link to publication in University of Groningen/UMCG research database

Citation for published version (APA):

Copyright
Other than for strictly personal use, it is not permitted to download or to forward/distribute the text or part of it without the consent of the author(s) and/or copyright holder(s), unless the work is under an open content license (like Creative Commons).

Take-down policy
If you believe that this document breaches copyright please contact us providing details, and we will remove access to the work immediately and investigate your claim.

Downloaded from the University of Groningen/UMCG research database (Pure): http://www.rug.nl/research/portal. For technical reasons the number of authors shown on this cover page is limited to 10 maximum.
The in vitro response of tissue stem cells to irradiation with different LETs

Peter W. Nagle*
Nynke A. Hosper*
Emily M. Ploeg
Marc-Jan van Goethem
Sytze Brandenburg
Johannes A. Langendijk
Roland K. Chiu
Robert P. Coppes

*These authors contributed equally

(International Journal of Radiation Oncology Biology Physics
2016;95(1):103-11)
SUMMARY

The difference in response of normal tissue to different radiation qualities is poorly understood. Recently, we developed a method to culture/expand salivary gland stem cells allowing assessment of the in vitro response of tissue stem cells to different radiation qualities. Our results indicate differences in response of stem cells to photons and carbon ions at different LETs and relative resistance to particle irradiation of salivary gland stem cells compared to immortalized HSG cells.

ABSTRACT

Purpose: Reduction of dose, irradiated volume and sensitivity of especially normal tissue stem cells is needed to advance radiotherapy. This could be obtained with the use of particles for radiotherapy. However, the radiation response of normal tissue stem cells is still an enigma. Therefore, in this study we developed a model to investigate the in vitro response of stem cells to particle irradiation.

Methods and Materials: We used the immortalized human salivary gland (HSG) cell line resembling salivary gland (SG) cells, to translate radiation response in 2D to 3D. This response was subsequently translated to the response of SG stem cells (SGSC). Dispersed single cells were irradiated with photons or carbon ions at different LET (48.76±2.16, 149.9±10.8 and 189±15 keV/µm). Subsequently 2D or 3D clonogenicity was determined by counting colonies or secondary stem cell derived spheres in Matrigel. γH2AX immunostaining was used to assess DNA double strand break repair.

Results: The 2D response of HSG cells showed a similar increase in dose response to increasing higher LET irradiation as other cell lines. The 3D response of HSG cells to increasing LET irradiation was reduced compared to the 2D response. Finally, the response of mouse SGSC to photons was similar to the 3D response of HSG cells. The response to higher LET irradiation was reduced in the stem cells.

Conclusions: Mouse SGSC radiosensitivity seems reduced to higher LET radiation when compared to transformed HSG cells. The developed model to assess the radiation response of SGSC offers novel possibilities to study radiation response of normal tissue in vitro.
INTRODUCTION

Radiotherapy with photons (+/- surgery, chemotherapy) is one of the most common and effective cancer treatment modalities but may result in side effects to the normal tissue such as radiation-induced fibrosis (1), xerostomia (2), cardiopulmonary disease (3), and radiation-induced liver damage (4). The risk of these side effects is not only related to the dose and volume of normal tissue which is co-irradiated when the tumor is being treated, but also to the location of the stem cells (5). Therefore, reducing dose-volume parameters and assessing the localization and radiosensitivity of stem cells are currently the focus of advancing radiotherapy.

It is important to understand the radiation response of tissue/adult stem cells (ASC), as these are for a major part responsible for the long-term regeneration of tissue (5). However, it is complicated to study ASC after irradiation in vivo and these cells cannot be cultured in two-dimensional (2D) systems. Recently it has become possible to culture ASC types in three-dimensional (3D) culture systems, such as stem cells isolated from intestines (6), liver (7), and salivary glands (8). The use of 3D culture systems would allow for in vitro studies of ASC in response to radiation. Recently, our group developed a 3D culture system of normal tissue, as spheres (a cluster of cells growing in all directions greater than 50μm in diameter) and organoids, containing stem cells (8). This system comprises multiple cell types (including stem cells) and represents an unprecedented opportunity to obtain knowledge about mechanisms of normal tissue response to irradiation.

While a large number of normal tissue cell types (such as fibroblasts (9), adipose (10), keratinocytes (11) and hematopoietic stem cells (12-14)) have been used in in vitro radiation studies, no studies used stem cells involved in tissue regeneration post-irradiation. A significant development in in vitro cell culturing studies was the development of 3D culture systems, used for studies of cancer models and treatment (15-17), drug discovery (18) and radiation therapy (19). 3D cell culture models are more physiologically relevant and offer a more realistic environment. Cells grown in 2D cultures are generally flat, however in 3D cultures cells are rounder and divide to form spheroid structures consisting of multiple cells originating from single cells. In 3D cell systems different cell-cell and cell-matrix interactions exist, while the components of the extra-cellular matrix of the 3D membrane itself also plays a role in radioresistance compared to 2D culture systems (15, 18, 19).
Further potential improvements of radiotherapy include charged particle therapy allowing improved tumor targeting and dose/volume reduction sparing normal tissue (2, 20, 21). However, the limited use of particle therapy limits our current knowledge of its biological effects. Although in vitro studies found important differences with photon treatment (22), the majority of these studies have looked at tumorigenic cell lines in a 2D environment. Very little is known about the effects of particle therapy on normal tissue stem cells.

Therefore, we developed a 3D culture model for the study of the radiation response of ASC useable for both photon and particle irradiations. We used a human submandibular salivary gland cell line (HSG) (23) derived from a patient that received external irradiation, which still has differentiation potential (24), to optimize our 3D culture system for carbon ion irradiations. We compared the radiation effects that culturing in 3D has following carbon ion irradiations with 2D culturing, both in the plateau and Spread Out Bragg Peak (SOBP) regions of the beam. Finally, using our salivary gland culture system, we are the first to show the survival of tissue specific stem cells in response to radiation treatment, of both photon and carbon ions. This may represent a novel model for studying normal tissue response to irradiation.

METHODS AND MATERIALS

Cell line culturing

The human salivary gland (HSG) cell line was cultured in Dulbecco’s Modified Eagle medium (DMEM) – F12 (1:1) (Gibco, Life Technologies) supplemented with 10% fetal bovine serum and 1% Penicillin/Streptomycin.

For 3D cultures, cells were detached using Trypsin-EDTA 0.05% for 5 minutes at 37°C and counted. The number of cells was adjusted to 200,000 cells/mL. Suspension was added to Basement Membrane Matrigel (Matrigel)(BD Biosciences, 354234) at a ratio of 1:2 (25μl Cell suspension:50μl Matrigel) and seeded in 12-well tissue culture plates. One week after seeding, gels were incubated with dispase (1mg/mL in culturing media; Gibco, Life Technologies) for 45-60 minutes at 37°C to dissolve the Matrigel, spheres were counted, and processed into single cells using Trypsin-EDTA 0.05%, and reseeded for irradiation treatment.
Isolation of salivary gland cells

SGs were dissected from 8-12 week old female C57BL/6 mice (Harlan, The Netherlands). SG cells were isolated and cultured to form spheres as described previously (8, 25, 26). For details see supplementary methods.

Irradiation treatment

Photon irradiation was performed with a 137Cesium source (IBL 637 Cesium-137 γ-ray machine) with a dose rate of 0.59 Gy/min, or with x-rays performed using the Xstrahl 200 X-ray Therapy System with a dose rate of 0.52 Gy/min. Carbon ion irradiations were described previously (22, 27), see supplementary methods. For irradiations in Matrigel a 3.5 mm long SOBP with a diameter of 30 mm was developed which resulted in a dose averaged LET of 149.9 +/- 10 keV/um at the center of the Matrigel samples, while the +/- represents the LET at the anterior and posterior of the gel. Photon irradiations of 2D cultures HSG cells were performed in 60mm dishes, while carbon ion irradiations were performed in 12-well culture plates, due to the physical constraints of the beam. Specifics regarding cell densities and time of irradiation post-seeding are mentioned in specific assays.

Clonogenic survival assays

Clonogenic survival assays were performed similar to the method described in (28). For details see supplementary methods. Plates were 70-80% confluent at time of irradiation.

To determine the sensitivity of cells cultured in 3D following irradiation, a modified 3D survival assay was performed. Cells were seeded as single cells in Matrigel as described above 2 hours pre-irradiation with 0-8 Gy. For HSG cells irradiated with 0-2 Gy were seeded at 5x10³ cells per well, while cells irradiated with 4 or 8 Gy were seeded at 1 x 10⁴ cells per well, but with equal volumes of Matrigel per well. mSGSCs were irradiated and re-plated at a density of 2x10⁴ cells per well (0-2 Gy) or 6x10⁴ cells per well (4-8 Gy). One-week post-irradiation, spheres and cells were counted. Survival was calculated as follows:

\[ \text{Sphere forming potential} = \frac{\text{Number of spheres harvested}}{\text{Cells seeded}} \times 100 \]

\[ \text{Surviving fraction} = \frac{\text{Sphere forming potential treated}}{\text{Sphere forming potential at 0 Gy}} \]
**Immunofluorescent microscopy**

2 days prior to irradiation for immunofluorescent microscopy of 2D cultures, cells were seeded in glass bottomed 12-well tissue culture plates (*In Vitro* Scientific, p12-1.5H-N). At specified time points post-irradiation, cells were fixed for 15 minutes in 2% paraformaldehyde and permeabilized in 0.2% Triton X-100 for 10 minutes.

For immunofluorescent microscopy of 3D cultures, 4x10^4 single cells were seeded in Matrigel 2 hours pre-irradiation. Dispase was added 30 minutes prior to stated time points. Cells were spun for 30 seconds, and washed with PBS. Cells were then resuspended in 2% paraformaldehyde, placed on Adhesion slides (Marienfeld), fixed for 15 minutes and permeabilized for 10 minutes.

All samples were incubated overnight at 4°C with primary antibodies (anti-phospho-Histone H2A.X (Ser139), clone JBW301 (1:500; Millipore, 05-636, mouse) and anti-53BP1 (H-300) (1:500; Santa Cruz, sc-22760, rabbit)), followed by incubation at room temperature with secondary antibodies (Alexa Fluor 488 (1:800; Life technologies, A11001, goat anti-mouse) and Alexa Fluor 594 (1:800; Life technologies, A110012, goat anti-rabbit)) for 90 minutes. Nuclear staining was performed using Hoechst 33342 (Molecular Probes, Life Technologies). Imaging was performed using TissueFAxs (Tissuegnostics), foci were analysed and counted using ImageJ.

**Data analysis**

All values are represented as mean ± standard error of the mean (SEM) (**p<0.01, *p<0.05) of at least three independent experiments. Student’s t-test was used for testing statistical significance using GraphPad Prism (GraphPad software) software. If error bars are not visible, they are smaller than data labels.
RESULTS

To study the *in vitro* response of SGSC we have to assess this in 3D culture. However, to relate 2D models with a 3D model for SGSC irradiation, we first determined the 2D radiation response of HSG cells to photons or carbon ions of various linear energy transfer (LET). As expected, increasing LET of radiation resulted in decreased survival of HSG cells at the same dose (Figure 1). In line with the literature (20, 22) we observed an RBE10 of approximately 1.72, 2.59 and 3.53 for carbon ion LET of 48.76±2.16 keV/µm, 149.9±10.8 keV/µm and 189±15 keV/µm at 10% survival, respectively.

![Figure 1: Survival of 2D cultured HSG cells in response to photons, Carbon ion 47.86 keV/µm, 149.9 keV/µm and 189 keV/µm, as measured by clonogenic survival assays. Error bars represent S.E.M., N=3.](image)

Next we investigated whether culturing in 3D affects proliferation. No significant differences in population doublings in 2D (5.37±0.2) compared to 3D (5.15±0.26) were found at 7 days (Figure 2A). This suggests that our 3D environment does not affect cell proliferation.

By performing a modified clonogenic assay, based on the efficiency of irradiated single cells to form spheres (Figure 2C), we determined the survival of HSG cells cultured in 3D in response to high and low LET irradiations (Figure 2D). Similar to culturing in 2D, we observed that increasing LET at the same dose resulted in an increased radiosensitivity in HSG cells, but in 3D cultured HSG cells were more radioresistant than 2D HSG cells (Figure 1), as has been demonstrated for other cell lines (19). Here the RBE10 of HSG cells were 1.34 and 3.03 for carbon ions at 48.76±2.16 keV/µm and 149.9±10.8 keV/µm, respectively. Due to the physical nature of the beam it was not possible to perform carbon ion irradiations using the highest LET (189±15 keV/µm).
To exclude the role of (intra-spheroid) oxygen diffusion as a factor for altered survival in 3D compared to 2D, we performed irradiations at various stages of HSG sphere development. HSG cells were photon-irradiated as single cells in media and subsequently seeded into Matrigel, single cells already seeded in Matrigel or as 6 day old spheres in Matrigel which were dissociated and reseeded 2 days later in Matrigel. We found that the levels of survival were not affected (Supplementary Figure 1), indicating oxygen diffusion levels at time of irradiation are unlikely to affect the HSG survival response to irradiation in the current set-up, however it must be acknowledged there may be other environmental effects which are not detected by our assay.

Next, we assessed the levels of DNA double strand breaks (DSB) (29-31) in HSG cells following irradiation with 1 Gy and subsequent repair using immunofluorescent microscopy for γH2AX (Figure 3). At 24 hours post 1 Gy photon irradiation still 33.6±3.4% of cells were positive for γH2AX (≥3 foci) following photon irradiation, whereas significantly more were positive following 149.9±10.8 keV/µm and 189±15 keV/µm at 41.4±3.4% (p=0.0486) and 49.1±6.2% (p=0.0055), respectively were found following 1 Gy carbon ions. 48.76 keV/µm irradiations showed no difference from photons for residual DSBs, which was consistent with the survival data at 1 Gy. So, as expected a significantly higher proportion of cells remained positive for DSB post higher LET irradiations. These observations are in line with previous studies on other cell lines which have shown that persistent DNA damage 24 hours after irradiation is a key factor in determining cell survival (30).
Figure 3: Levels of double stranded DNA breaks were studied 30 minutes, 4 hours and 24 hours post-irradiation of 2D cultures by immunofluorescent microscopy for (A) γH2AX. (B) The levels of persistent damage 24 hours post-irradiation in 2D. Error bars represent S.E.M., N≥3.
Next the levels of DSBs in 3D cultured cells following 1 Gy irradiation was assessed. We found that residual γH2AX in the 149.9±10.8 keV/µm was significantly higher than photons (62.9±4.6% to 37.9±1.5%, p=0.0067), however there was no difference between photons and 48.76±2.16 keV/µm (43±5.7%) (Figure 4). This was not surprising, as in terms of survival 1 Gy photons was comparable to 48.76±2.16 keV/µm carbon ions, while survival following 149.9±10.8 keV/µm carbon ions was significantly decreased.

Figure 4: (A) Representative images of γH2AX immunofluorescent microscopy following 3D irradiations. (B) Quantification of residual damage 24 hours post-irradiation in Matrigel.
Finally, we tested the response of mSGSC in our 3D culturing model. In salispheres (spheres formed from salivary gland derived cells (25)) cultured from dispersed mouse SG cells, only the stem/progenitor cells can give rise to secondary spheres, all other cells will die off even without irradiation (8). Here we irradiated single salisphere cells and determined the sphere forming efficiencies of the cells after passaging (Figure 5A) as a representative of surviving number of mSGSCs seven days following irradiation. Sphere forming efficiencies were then normalized to 0 Gy to calculate the surviving fraction (Figure 5B). Following photon irradiation the isolated mSGSCs displayed a clear dose-response relationship, with survival decreasing from 68.3±12.6% for 1 Gy photons to 15.5±1.8% survival at 8 Gy. Irradiating with carbon ions (both 48.76±2.16 keV/µm and 149.9±10.8 keV/µm) also resulted in a clear dose-response curve, albeit with a stronger response to carbon ions, with a survival of 45.3±4.2% following 1 Gy 149.9±10.8 keV/µm carbon ions and only 2.4±0.2% survival after 8 Gy. It was necessary to extrapolate the photon survival curve, in order to determine a RBE10 for isolated mSGSCs. The calculated RBE10 were 1.72 for 48.76±2.16 keV/µm and 2.90 for 149.9±10.8 keV/µm, similar to HSG cells irradiated in 3D cultures. These data suggest that, similar to cell lines, the radiosensitivity of SGSC increases with increasing LETs.

When we compared the response of HSG cells in 3D cultures with that of mSGSCs (Figure 5C, D, E), we found that HSG cells and mSGSCs show a similar pattern of radiosensitivity to photons. As mentioned previously, the HSG cell line is transformed, however it is non-tumorigenic (23). These results may suggest that HSG cells may be more similar to a normal stem cell containing population than previously thought, particularly in response to irradiation. However, when we compared the response of the same cells to carbon ion irradiations, we found that the mSGSCs are less radiosensitive to both 48.76±2.16 keV/µm (p=0.0314 at 4 Gy, p=0.0415 at 8 Gy) and 149.9±10.8 keV/µm (p=0.0149 at 4 Gy, p=0.0057 at 8 Gy) than HSG cells. This could suggest that the stem cells themselves might be a key factor to a potential increased radioresistance to higher LET irradiations.
Figure 5: (A) Schematic overview of isolation, expansion and irradiation of mSGSCs. (B) Surviving fraction of isolated mSGSCs following photon and Carbon ion (48.76 keV/µm and 149.9 keV/µm) irradiations. (C, D, E) Comparison of responses of HSG cells grown in 2D versus 3D versus mSGSCs in response to B) photons, C) carbon ions 48.76 keV/µm and D) 149.9keV/µm. Error bars represent S.E.M., N≥3.
DISCUSSION

We developed a model derived from our previously established mSGSCs cultures (8) for assessing normal tissue response following irradiation with modalities with differing LETs. We showed a similar radiosensitivity to photon irradiation of mSGSCs and 3D cultured HSG cells. Interestingly a potential reduced sensitivity of mSGSCs to higher LET carbon ions was observed. We believe this model may represent a novel way to obtain knowledge on normal tissue side effects.

Our mSGSCs model is a 3D culture (8). Therefore, to optimize our model for irradiation, we used the transformed epithelial, non-neoplastic HSG cell line (23) which can be cultured both in 2D and as 3D spheres (24) similar to our salispheres. As expected, in 2D cultures a clear dose-response following increasing doses of photon irradiation was seen. Following carbon ion irradiations this dose-response effect was even more evident with increasing LET. The RBE values were within the range found for other cell lines following carbon ion irradiations (20, 22). Due to the more complex nature of DNA damage induced by particle therapy (32), this was not unexpected as this damage is slower to repair and therefore more likely to remain unrepaired 24 hours post-irradiation. It has been shown that persistent damage 24 hours post-irradiation is critical in cell lethality (30). Indeed, the percentage of cells that remained positive for DNA DSBs 24 hours post-irradiation was higher after carbon irradiation, which correlates with an increased radiosensitivity with increasing LET. The 2D survival data and the DNA residual damage show that this is a strong model for furthering our studies towards 3D studies. Having determined the phenotypic response of HSG cells to irradiation when cultured in 2D conditions, we then determined the effects that culturing these cells under 3D conditions has following irradiation. We showed similar radiosensitivity reduction under 3D culture conditions for HSG cells when compared to other cell lines (19, 33). Furthermore, we found that the 3D cultured cells showed a similar phenotypic response to differing LET irradiations as in 2D conditions. Again in line with previous studies (30) and similar to our 2D findings, a significantly higher level of γH2AX with increasing LET correlated with an increased radiosensitivity. The levels of residual damage were perhaps higher than expected when compared to the levels of survival at 1 Gy, which may suggest that a later time point post-irradiation may be more insightful in terms of final clonogenic survival.

Finally, we irradiated mSGSCs which had been cultured in our recently developed culture system for normal tissue(8). Because this system consists of several cell types, including stem cells, we believe this is an ideal model for studying normal tissue side effects following irradiation. We found that the
mSGSCs displayed increased sensitivity to higher LETs. We found that our mSGSCs showed a similar radiosensitivity to HSG cells in response to photons, this may indicate that HSG cells are a strong model for studying normal tissue response to photons. In response to increasing LET the mSGSCs showed an increased radiosensitivity in comparison to HSG cells. This suggests that the stem cells might be more radioresistant to increasing LETs than photons.

This is the first in vitro study to irradiate mSGSCs also using high LET carbon ions. There are some limitations to our model. Firstly, in a true in vivo environment ASCs reside in a quiescent state and only divide in response to certain cues, for example to regenerate damaged tissue (34). However, in our system SGSC are driven towards proliferation (8), thereby possibly altering the response to irradiation. It is also important to note that environmental factors, such as inflammatory processes, macrophages and secreted cytokines (such as TGF-β (35)) in response to radiation are not present in our system, addition of these factors may alter the response.

However, our model still represents a breakthrough method for studying normal tissue response in vitro, showing important differences in the response of ASC to high and low LET radiation modalities and could be used to study radiation induced normal effects. We believe that the use of ASCs gives a better understanding of normal tissue response than other 3D models currently available. A recent study has been performed to investigate the response of adipose-derived stem cells irradiated under 3D conditions (10) in which the authors also observed an increased resistance under 3D conditions and differences between LET irradiations modalities. However, these are not tissue-specific stem cells and therefore we believe our model may be an improved alternative to study normal tissue damage following irradiation. In the future, the use of patient derived ASC could determine differences in individual patient specific responses and contribute to patient specific treatment planning.
REFERENCES


SUPPLEMENTARY METHODS AND MATERIALS

Isolation of salivary gland cells
In short, salivary glands were first mechanically disrupted, followed by enzymatic digestion using digestion enzymes collagenase type II (Gibco) and hyaluronidase (Sigma Aldrich). After filtering, cells were seeded into 12 wells plates in DMEM-F12 medium (Gibco) containing 1% penicillin/streptomycin (Gibco), glutamax (2mM, Gibco), epidermal growth factor (20ng/ml, Sigma Aldrich), fibroblast growth factor-2 (20ng/ml, Peprotech), 1% N2 supplement (Gibco), insulin (10 μg ml-1, Sigma) and dexamethasone (1μM, Sigma). After three days, primary spheres (passage 0) were disrupted using 0.05% trypsin-EDTA (Gibco) into single cells and reseeded at a density of 10,000 cells/well in Matrigel and medium supplemented with ROCK1 inhibitor; Y-27632 (Sigma-Aldrich) was added to the gels (passage 1).

Clonogenic survival assays
All irradiations were performed on 80% confluent cells. Photon irradiations were performed in 6-cm dishes, while carbon ion irradiations were performed in sealed 12-well plates as described in (1). Following irradiation the cells were detached from the plates by incubating for 5 minutes with trypsin/0.05% EDTA (Gibco). Cells were counted and then seeded in triplicate in 6-cm dishes at dose dependent concentrations. After 10-14 days colonies were fixed and stained by incubating with 0.1% (w/v) Coomassie Brilliant Blue, 50% methanol, and 10% acetic acid for 30-60 minutes and colonies containing ≥50 cells were counted. All experiments were normalized for plate efficiency of 0 Gy control.

Carbon ion irradiations
C-ion irradiation is performed with a (high LET) 90 mega electron volt per nucleon (MeV/u) carbon ions in a 1.3mm long SOBP with a diameter of 30mm, allowing accurate irradiation of the cells. At the position of the sample, the track averaged LET was 132 ± 10 keV/μm were the dose average was 189 ± 15 keV/μm.
SUPPLEMENTARY FIGURES

Figure S 1: Comparison of survival of 3D HSG cells irradiated under different conditions. Cells were irradiated either as single cells in suspension and subsequently seeded (IRR. in suspension), as single cells already seeded in Matrigel (Early IRR. in Matrigel), or as 6 day old spheres which were dissociated to single cells and reseeded two days later (Late IRR. in Matrigel).