doi:10.1017/S1431927617007243
Microsc. Microanal. 23 (Suppl 1), 2017

Effect of γ irradiation on autophagic flux in glioblastoma cells that express LC3B-eGFP-mCherry

Linda S. Yasui[1], Van Bui[1], Anita Latgnotha[1].

[1.] Northern Illinois University, Department of Biological Sciences, DeKalb, IL. USA.

Glioblastoma is a deadly human brain tumor. Prospects for survival beyond a year are dismal because the tumor is almost certain to recur as its cells are highly resistant to radiotherapy and chemotherapy [1]. Pro-apoptotic strategies have not been fully successful in enhancing radiation-induced cell death mainly because GBM cells are intensely resistant to apoptosis [2]. In order to improve prospects for individuals having this deadly disease, we propose to subvert radiation resistance in GBM by focusing on a promising metabolic target for therapeutic intervention termed autophagy. Autophagy is a known cell death mechanism in GBM cells [3] [4] which converts metabolic perturbations into vital or lethal events via a multi-step, highly regulated cellular degradation process [5]. Since autophagy acts as a cell death checkpoint, we propose to investigate the entire process of autophagy (autophagic flux). Autophagic flux describes a progression through the initial formation of autophagosomes that sequester, package and traffic damaged or old macromolecules and organelles to lysosomes for hydrolytic digestion where ultimately, the metabolic building blocks that the cell can reuse are produced. Autophagic flux has been identified by us to correlate with radiation-induced cell death in GBM cells [6].

The human U87 GBM cells used for these experiments were cultured as previously described by us [6]. Autophagic flux was measured using a tandem monomeric fluorescent LC3B protein (mCherry-eGFP-tagged LC3 or tfLC3). Stable clones of U87 cells that express the autophagy marker protein, tfLC3 were generated by us [6]. The autophagy biomarker protein, LC3B co-localizes 2 fluorescent proteins, eGFP and mCherry, to autophagosome organelles. Co-localization of both the red and green fluorescence dyes results in yellow fluorescence indicating early stage autophagosome organelles. During late events of autophagy when autophagosomes fuse with the lysosomes, the acidic autophagolysosome organelles fluoresce red because the green fluorescence is quenched due to pKa differences between eGFP and mCherry (pKa values of 6.5 and less than 4.5, respectively). Thus, the appearance of red or yellow fluorescent autophagosome organelles permits estimation of autophagic flux that is easily achieved in essentially native conditions with the use of multiple time-points [7]. In this system, when the number of both yellow and green puntae increases from the basal level, autophagic flux is increased; however, if autophagosome maturation into autolysosomes is blocked, an increase in number of just the yellow punctae is observed without a concomitant increase in red punctae.

The presence of red or yellow stained punctae or autophagosomes was visualized for cells cultured on coverslips for 0, 1, 3, 5 or 7 days post irradiation or sham exposure to ^{137}Cs irradiation. Imaging parameters were optimized. Minimal refractive index (RI) mismatch was achieved using tissue culture medium as a mounting medium because the RI of individual cells equals 1.36 and the RI of typical tissue culture medium is close to that at 1.335, leading to an optical path difference of 0.125 µm or about 1/4 of a wavelength. Cells were imaged using a Zeiss LSM 5 Pascal CLSM (Carl Zeiss Microscopes, Thornwood, NY, USA) using a differential interference contrast (DIC) plan neofluor 40X, NA 1.30, oil immersion objective lens. The pinhole was set to 1 airy unit. Optical sections (0.5 µm) of cells were acquired at 1024 X 1024 pixels giving a x-y pixel dimension of 0.22 µm, close to the limit of resolution of the light microscope [8]. Image files were saved as .lsm files and imported into LSM 5 Image

Browser (http://www.zeiss.com/microscopy/en_us/downloads/lsm-5-series.html) for analysis. The imported image was opened as a split x-y screen (Fig. 1a). Data for a single cell was extracted from the field of view by encircling a cells using the overlay tool. The single cell was then analyzed with the transmitted light illumination turned off to better see red, green or yellow puncta. Finally, a single slice through the z-stack of images was selected on the basis of the clarity of puncta. Red, green and yellow puncta were scored and this data was collected in an Excel spread sheet. The fraction of yellow or red puncta relative to the total number of red and green puncta was plotted (Fig. 1b and 1c). Thirty images per treatment were collected. As can be seen in Fig. 1b, no difference in autophagic flux was observed between day 1, 3 or 7. For data in Fig. 1c, ANOVA analysis indicated a significant difference between the means of the irradiated samples compared to the unirradiated sample. A post-hoc Tukey test specifies a significant difference between D1 vs D1 10 Gy ($p < 0.0001$), D1 vs D3 10 Gy ($p=0.0012$), and D1 vs D7 10 Gy ($p=0.0283$).

These data support our conclusion that establishment of altered autophagic flux can be a useful biomarker for metabolic stress and provide metabolic context for radiation sensitization to ionizing radiation.

References:

[1] TF Cloughesy, WK Cavenee, PS Mischel. 2014. Annual Review of Pathology **9** (2014) p.1-25.
[2] F Lefranc, J Brotchi, R Kiss. Journal of Clinical Oncology **23** (2005) p. 2411-2422.
[3] KC Yao et al. Journal of Neurosurgery **98** (2003) p. 378–384.
[4]T Kanzawa et al. Cell Death and Differentiation **11** (2004) p. 448-57.
[5] DR Green, L Galluzzi, G Kroemer. Science **345** (2014) p. 1-11.
[6] LS Yasui et al. International Journal of Radiation Biology **92** (2016) p. 665-678.
[7] D Klionsky et al. Autophagy **12** (2016) p. 1-222
[8] LS Yasui et al. Radiation Research **167** (2007) p.167–175.

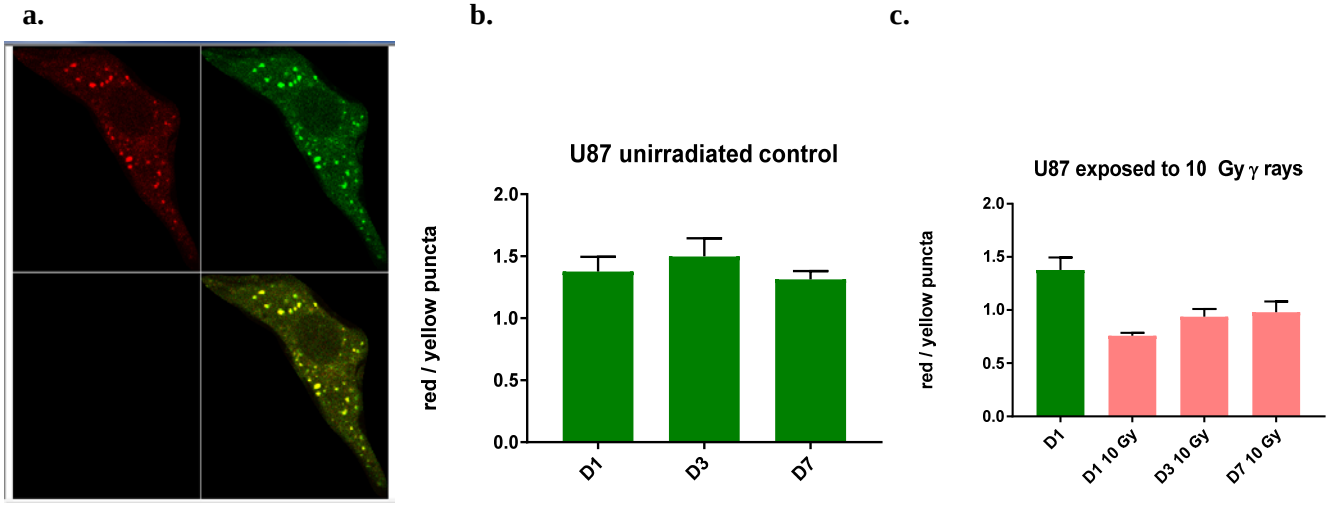

Figure 1. Autophagic flux was measured using the tanden autophagy marker stably expressed in U87 cells. A representative split X-Y screen image of a z-slice through a U87 cell expressing tfLC3 is shown in a. A comparison of autophagic flux for unirradiated cells with irradiated cells imaged 1, 3 or 7 days after exposure to 10 Gy γ rays shows a significant difference in the means. Mean values +/- 1 SEM are presented in the figure.

https://doi.org/10.1017/S1431927617007243 Published online by Cambridge University Press