The induction of DNA damage signalling/repair responses in mammalian cells by near infrared femtosecond laser pulses

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Introduction

Exposing cells to ionising radiation (IR) causes a variety of random DNA lesions including base damage, single stand breaks (SSBs), doubles strand breaks (DSBs) and clustered lesions; a specific feature of IR when two or more lesions form within one to two helical turns of DNA[1,2]. DSBs are the most deleterious lesion formed and are repaired by two main repair pathways, nonhomologous end joining (NHEJ), prevalent throughout all phases of the cell cycle, and homologous recombination (HR) prevalent during late S-phase and G_2-phase of the cell cycle [3]. Failure of cells to accurately and efficiently repair DNA damage results in mutations, genome instability and cancer.

We have previously demonstrated the induction of DNA damage through a high powered near infrared (NIR) femtosecond laser pulse within a femtolitre volume of the nucleus. The laser was tuned to the previously optimised wavelength 730 nm where there are no significant cellular absorbers. Multiphoton processes occurring at the focal point of the laser microbeam induce DSBs and complex lesions. A core histone molecule, H2AX is phosphorylated in response to DSB formation (γ-H2AX) and correlates with the number of DSBs formed, as confirmed by comet assay [4]. Using the laser microbeam technique co-localisation of signalling/repair proteins with a marker of DSBs, γ-H2AX, can be studied to determine the kinetics of the early stages of DNA damage signalling/repair in mammalian cells.

RAD51 is a repair protein involved in the HR pathway. It is involved in recruitment of the sister chromatid to be used as a template for repair and is therefore prevalent in cells in the latter stages of S-phase and G_2-phase. p53 binding protein 1 (53BP1) has been identified as a protein involved in the early stages of DNA damage repair. It has been suggested that 53BP1 may be involved in the activation of ATM (the major kinase involved in phosphorylation of H2AX following IR) and also BRCA1 and RAD51 [5].

Previous work using this novel laser microbeam technique provided information regarding laser microbeam induction of DSBs and preliminary information regarding co-localisation of various signalling/repair proteins including ataxia telangiectasia mutated (ATM), RAD51 and 53BP1[6]. We have recently gathered more detailed information regarding the co-localisation of RAD51 and 53BP1 with γ-H2AX and the time course of repair following damage induction by the NIR microbeam.

Methods

Cell Culture

Chinese hamster V79-4 cells were cultured in minimum essential medium (MEM) supplemented with 10% foetal calf serum (FCS), 100 µg/ml penicillin streptomycin and 2mg/ml L-glutamine at 37ºC with 5% CO_2 in air.

Raster Scanning of Mammalian Cells

A titanium sapphire (Ti:Sa) laser (Mira 900, Coherent Inc., USA) tuned to 730 nm was focused through an ×40, numerical aperture 0.9 microscope objective of an inverted microscope (TE2000, Nikon). A customised computer software programme (LabView™) operated the automated microscope stage in the x-plane and the y-plane at a step size of 12 µm and with scanning covering an area of 0.25 cm × 0.5 cm.

1 mg/ml of Hoechst dye was added to the cells and incubated for 10 min at 4°C. The dishes were cooled to 10°C throughout irradiation with raster scanning. Following raster scanning, the cell medium was replaced with medium warmed to 37°C and then incubated at 37ºC with 5% CO_2 in air for differing repair times.

Immunofluorescence

Cells were fixed in 3% paraformaldehyde in MEM at 4ºC for 10 min followed by washing with 1 ml MEM and permeabilisation using 500 µl of 1% Triton X-100 for 10 min. Cells were then rinsed with 1 ml MEM and blocked for 1 h at room temperature in 500 µl of 1% bovine serum albumin (BSA) and 1% fish skin gelatin (FSG) in MEM. Cells were then rinsed with 1 ml MEM and 200 µl of the primary antibody to the protein of interest, in 1% BSA and 1% FSG was added overnight at 4°C followed by a rinse with 1 ml MEM. Cells were then labeled with 200 µl of secondary antibody (Fluorescein isothiocyanate, FITC or cyanine 5, Cy5) for 1 h at room temperature. Cells were rinsed in 1 ml MEM and mounted in Vectashield® antifading medium. Cells were then viewed using a Biorad MRC600 confocal microscope with an × 60-oil objective.

Antibodies

The antibodies used in the experiments were purchased from the following companies without any processing. Anti-phospho-Histone H2A.X (Ser139), clone JBW301, 05-636 and Anti-phospho-H2A.X (Ser139), 07-164, Upstate, Hampshire, UK. ·-RAD51 PC130, rabbit pAb., Calbiochem, Nottingham, UK. Fluor (FITC) AffiniPure F(ab')2 Frag Goat Anti-Mouse and Cy5 AffiniPure F(ab')2 Fragment Goat Anti-Rabbit, Stratech Scientific Unit, Cambridge, UK. Rabbit anti-53BP1 (BL182) affinity purified A300-273A, Universal Biologicals (Cambridge) Ltd, Cambridge, UK.

Results

Co-localisation of RAD51 with γ-H2AX

RAD51 was seen to co-localise with γ-H2AX although the response of RAD51 to DSBs is significantly later than that of γ-H2AX. γ-H2AX was seen to peak approximately 30 min post-irradiation however, RAD51 levels peak approximately 180 min following irradiation with the laser

microbeam. This may reflect the possible handover from signalling proteins to repair proteins at the sites of DSBs (Fig. 1). RAD51 appears to be prevalent in approximately 50-70% of cells at earlier time points, which correlates with the number of cells expected to be in the S-phase and G_2/M-phases of the cell cycle as demonstrated by flow cytometry (data not shown). At latter stages of the repair process, RAD51 would be expected to increase due to the number of cells that become blocked in the G_2/M-phase before declining in response to repair of DSBs. At 24 h post-irradiation there is evidence of DSB persisting although the damage lines are becoming dispersed since more individual foci are seen reflecting the dispersion of the damage sites (Fig. 2). The co-localisation of RAD51 and γ-H2AX at sites of DNA damage persists at the 24 h repair time possibly reflecting the complexity of the damage produces by the laser (Fig. 2).

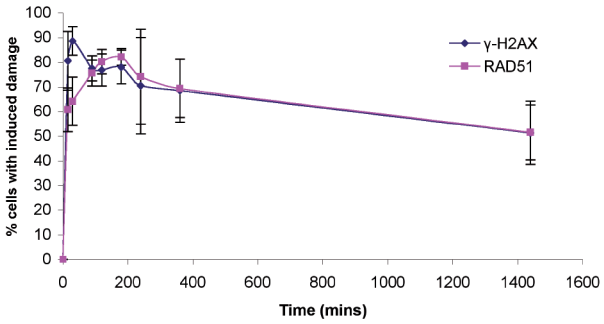
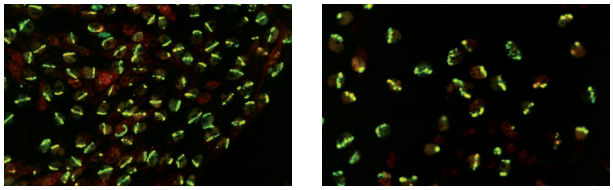


Figure 1. Occurrence of RAD51 and γ-H2AX in V79-4 cells over a 24 h repair time following laser microbeam irradiation. Mean of 3 experiments ±se.

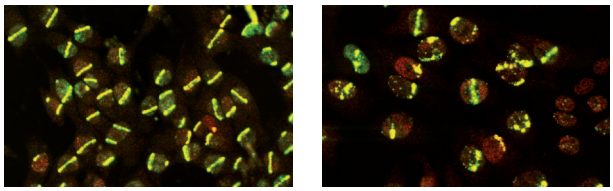


(a) (b)

Figure 2. Co-localisation of RAD51 and γ-H2AX (a) 360 min following laser microbeam irradiation of V79-4 cells (b) 24 h post laser microbeam irradiation of V79-4 cells. RAD51 shown as red Cy5, γ-H2AX shown as green FITC and co-localisation of both RAD51 and γ- H2AX shown as yellow.

Co-localisation of 53BP1 with γ-H2AX

53BP1 is recruited to sites of DSBs within 5 min post laser microbeam irradiation peaking at 30 min (Fig. 4). Following DSB formation, the repair time profile of 53BP1 closely follows that of γ-H2AX and the proteins are seen to co-localise (Fig. 3). The localisation of 53BP1 at sites of DNA damage persists 24 h post irradiation. It was also noticed that not only did the lines appear to dissipate indicating repair or diffusion of the damage sites but also they appeared thicker possibly due to the unwinding of the chromatin or the recruitment of larger numbers of 53BP1 and γ-H2AX protein. It was also noted that the nuclei of cells that still showed signs of DNA damage were much larger than cells that did not show signs of damage; this may be a reflection of cells being blocked at the G_2/M checkpoint at the 24 h repair time.



(a) (b)

Figure 3. Co-localisation of 53BP1 and γ-H2AX (a) 30 min following laser microbeam irradiation of V79-4 cells (b) 24 h following laser microbeam irradiation of V79-4 cells. 53BP1 shown as red Cy5, γ-H2AX shown as green FITC and co-localisation of both 53BP1 and γ-H2AX shown as yellow.

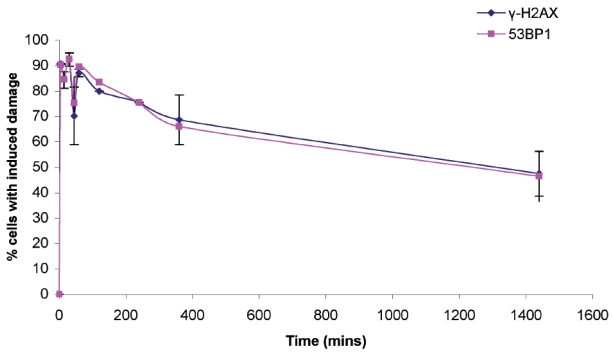


Figure 4. Occurrence of 53BP1 and γ-H2AX in V79-4 cells over a 24 h repair time post laser microbeam irradiation. Mean of 3 experiments ±se.

Conclusion

RAD51 and 53BP1 co-localise with γ-H2AX although they show very different responses to DNA damage induction with the NIR laser microbeam. 53BP1 has a rapid response to DSBs and appears within 5 min of irradiation as it is initially involved in the signaling/recruitment of repair proteins. RAD51 has a delayed response and appears in cells in S-phase and G_2/M-phases of the cell cycle due to its role in the HR pathway. RAD51 and 53BP1 are both present in cells 24 h post irradiation possibly as a result of the complexity of the DNA damage. Further studies into the kinetics of signaling/repair proteins in repair proficient and deficient cell lines will elucidate further the early response to DNA damage. An in house point revisiting software will be used to observe proteins in real time after exposure to NIR laser microbeam.

References

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