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Persistence of γ-H2AX and 53BP1 foci in proliferating and non-proliferating human mammary epithelial cells after exposure to γ-rays or iron ions.

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Abstract

Purpose: To investigate γ-H2AX (phosphorylated histone H2AX) and 53BP1 (tumour protein 53 binding protein No. 1) foci formation and removal in proliferating and non-proliferating human mammary epithelial cells (HMEC) after exposure to sparsely and densely ionizing radiation under different cell culture conditions.

Material and methods: HMEC cells were grown either as monolayers (2D) or in extracellular matrix to allow the formation of acinar structures *in vitro* (3D). Foci numbers were quantified by image analysis at various time points after exposure.

Results: Our results reveal that in non-proliferating cells under 2D and 3D cell culture conditions, iron-ion induced γ-H2AX foci were still present at 72 h after exposure, although 53BP1 foci returned to control levels at 48 h. In contrast in proliferating HMEC, both γ-H2AX and 53BP1 foci decreased to control levels during the 24–48 h time interval after irradiation under 2D conditions. Foci numbers decreased faster after γ-ray irradiation and returned to control levels by 12 h regardless of marker, cell proliferation status, and cell culture condition.

Conclusions: The disappearance of radiation induced γ-H2AX and 53BP1 foci in HMEC have different dynamics that depend on radiation quality and proliferation status. Notably, the general patterns do not depend on the cell culture condition (2D versus 3D). We speculate that the persistent γ-H2AX foci in iron-ion irradiated non-proliferating cells could be due to limited availability of double strand break (DSB) repair pathways in G0/G1-phase, or that repair of complex DSB requires replication or chromatin remodeling.

Introduction

The radiation risk from cosmic background radiation due to highly charged, high-energy (HZE) particles is relevant to long-term space travel such as a manned mission to Mars because it is not feasible to completely shield astronauts from this type of radiation (overview see (Cucinotta and Durante 2006; Durante and Cucinotta 2008)). Even though the fluence of protons is much higher in space than that of heavy ions, heavy ions have a greater ionization potential and contribute significantly to the total dose equivalent, with iron ions being one of the most important particles (Durante and Kronenberg 2005). Experimental studies indicate that densely ionizing radiation such as HZE particles may be a more potent carcinogen than sparsely ionizing X-rays or γ-rays, possibly due to the complexity of the induced DNA (deoxyribonucleic acid) damage (overview see (Held 2009)). Although ionizing radiation induces DNA base damage, single-strand breaks and double-strand breaks (DSB), the DNA double strand break is considered to be the most serious risk to genomic integrity.

We used γ-H2AX (phosphorylated histone H2AX) and 53BP1 (tumour protein 53 binding protein No. 1) nuclear foci to monitor DNA damage as a function of radiation quality in human mammary epithelial cells (HMEC). Both γ-H2AX and 53BP1 foci can be detected by immunostaining as microscopically visible nuclear domains at physiological relevant doses of ionizing radiation, which makes this technique a very useful tool to measure DSB induction and repair (review on γ-H2AX see (Pilch et al. 2003)). Rogakou et al. (1998, 1999) showed that DNA double strand breaks induce histone H2AX phosphorylation at serine 139 in the chromatin surrounding the DSB. Schultz et al. (2000) reported evidence that 53BP1 focus formation is specifically associated with agents that induce DSB and occur in similar numbers and the same

kinetics as γ-H2AX foci. Furthermore, Sedelnikova et al. (2002) reported a close correlation between ^{125}IdU induced DSB and γ-H2AX focus formation. Soutoglou et al. (2007) observed a 1:1 correlation between γ-H2AX foci and single DNA double stand breaks induced by the intron-encoded *Saccharomyces cerevisiae* endonuclease I (*ISce*I). Co-localization of 53BP1 with γ-H2AX foci support the assumption that they both occur at sites of DSB (Schultz et al. 2000).

It has been shown that high LET (linear energy transfer) radiation induces more residual DSB than low LET radiation in pulsed-field gel experiments (Stenerlöw et al. 2000; Rydberg et al. 2005), and more persistent foci in immunolocalization experiments (Karlsson and Stenerlöw 2004; Desai et al. 2005; Asaithamby et al. 2008). This is probably due to difficulties to repair complex DSB induced by high LET radiation involving multiple lesions spanning 10-20 nucleotides (Goodhead 1994). High LET radiation also produces DSB in close proximity to each other (Löbrich et al. 1996; Rydberg 1996), which may also affect repair ability and misrejoining probability (Rydberg et al. 2005). Most of the previously published repair studies were carried out using non-proliferating cells, and damage was followed for 24 h or less after irradiation. In our study we have extended the time of study up to 72 h and looked at both non-proliferating and proliferating HMEC. Various groups have demonstrated that three-dimensional growth and signals from the extracellular matrix are critical for normal epithelial cell morphogenesis and function (for an overview see (Bissell et al. 1982; Barcellos-Hoff et al. 1989; Kenny et al. 2007)). Therefore, we extended our studies of foci formation and resolution to HMEC embedded in an extracellular matrix (3D) (Debnath et al. 2003; Lee et al. 2007). To avoid bias during the foci counting and to improve the analysis of a substantial amount of data, foci numbers were quantified with image analysis programs developed by us (Parvin et al. 2007). We show that radiation-induced γ-H2AX and 53BP1 foci have different resolution dynamics that depend on

radiation quality and proliferation status, but that the general pattern seems to be independent of 2D (monolayer) versus 3D cell culture conditions.

Material and methods

Cell culture

The human mammary epithelial cell line MCF10A was derived from breast tissue from a 36-year-old woman with extensive fibrocystic disease (Soule et al. 1990). The cells were cultured in serum free MEBM (mammary epithelial basal medium with bicarbonate and phenol red; Cambrex, Charles City, IA, USA) supplemented with 100 ng/ml Cholera toxin (Sigma-Aldrich, St. Louis, MO, USA) and the SingleQuot®Kit (Cambrex) of supplements with growth factors and cytokines without gentamicin. Post-selection 184v (specimen 184 batch B) HMEC (provided by Dr. M. Stampfer, Lawrence Berkeley National Laboratory (LBNL)) are finite life-span human mammary epithelial cells derived from a reduction mammoplasty (Hammond et al. 1984). These HMEC were cultured in serum-free MEBM (without sodium-bicarbonate and phenol red; Cambrex) with 70 μg/ml bovine pituitary extract, 5 ng/ml human epidermal growth factor (EGF), 0.5 μg/ml hydrocortisone, 5 μg/ml insulin, 5 μg/ml transferrin, 10 μM isoproterenol (all supplements provided by Dr. M. Stampfer, LBNL), and 20 μM L-glutamine (Invitrogen, Carlsbad, CA, USA). All cells were tested for mycoplasma (Bionique Test Labs, Saranac Lake, NY, USA) and only cells that tested negative were used.

Cells were cultured in 25 cm^2 or 75 cm^2 tissue culture flasks (Invitrogen) and incubated at 95% humidity and 37° C under 5% CO_2 for MCF10A, or low CO_2 (0.2 – 0.8%; depending on incubator) for 184v. For passage, MCF10A cells were washed twice with 0.25% trypsin (University of California San Francisco (UCSF) cell culture facility, San Francisco, CA, USA) with 0.5 mM ethylenediaminetetraacetic acid (EDTA; Invitrogen) and incubated with 20% cell dissociation media (Sigma-Aldrich) in trypsin at 37° C. Trypsinization was stopped by adding an

equal volume of soybean trypsin inhibitor (1 mg/ml in medium; Sigma-Aldrich). 184v cells were washed twice with 0.05% trypsin (Sigma-Aldrich) with EDTA and incubated in the same for several minutes at 37°C. After the cells detached from the flask they were washed once in phosphate buffered saline (PBS; Invitrogen) before reseeded in appropriate numbers.

For immunostaining of 2D cultures, MCF10A cells were seeded in 8 well plastic chamber slides (Nalge Nunc International, Rochester, NY, USA) with 5000 cells per well 2 days in advance of exposure for cycling cell experiments and 4 days in advance of exposure for non-cycling cell experiments. Cells for non-cycling experiments were washed twice with PBS and transferred to medium without epidermal growth factor 2 days before irradiation.

For measuring foci in synchronized binucleated 184v cells, 8 well plastic chamber slides with 5000 cells per well were prepared 5 days before exposure. After two days cells were transferred to medium without EGF supplemented with 5 μg/ml of monoclonal antibody 225 (provided by Dr. M. Stampfer, LBNL (Stampfer et al. 1993)) directed against the EGF receptor (EGFR). Resting HMEC (184v) were released from the proliferation block by adding 25 ng/ml EGF to the cell culture medium 15, 17, 19 or 21 h before irradiation. Cytochalasin B (3 μg/ml; Sigma-Aldrich) was added 1 h after irradiation to prevent cytokinesis and cells were fixed 2 h later.

To examine cells released from proliferation block after irradiation, MCF10A cells were seeded in a similar manner as described above except no EGFR antibody was added to the cell culture medium and the cells were released 22 h after exposure or kept in a stationary state for the whole incubation repair time. For premature chromosome condensation (PCC) MCF10A cells were seeded in the same way as for the immunostaining samples except that 25 cm^2 tissue culture flasks were used. Cell numbers were chosen to reach similar cell seeding densities for both culture vessels (156,000 cells per T25 flask).

3D cultures of MCF10A cells were established using Cultrex (Trevigen, Gaithersburg, MD, USA), which is a soluble form of basement membrane purified from Engelbreth-Holm-Swarm (EHS) tumor (major components include laminin I, collagen IV, entactin, and heparin sulfate proteoglycan). Four-well coverslip glass bottom chamber slides (Nalge Nunc International) were coated with 60 µl of Cultrex (Trevigen) per well and incubated for at least 20 min at 37° C to solidify. 10.000 MCF10A cells in 0.4 ml medium were then seeded per well 7 days in advance of exposure. Cells were incubated for 30 min to allow them to settle down on the coating and then another 0.4 ml of 10% Cultrex in medium was added per well (final concentration of Cultrex was 5%). Cells were grown in complete medium for the first six days including EGF to allow for acini formation and functional polarization and were then transferred to medium without EGF the day before exposure.

Irradiation

Iron ion exposures with an energy of 968 MeV/amu (LET = 151 keV/µm) were performed at NASA (National Aeronautics and Space Administration) Space Radiation Laboratory (NSRL) at Brookhaven National Laboratory (BNL) (beamline details can be found at the NASA/BNL Space Radiation Program homepage at http://www.bnl.gov/medical/NASA/). The dose rate was 1 Gy/min, resulting in short acute exposure times of 0.5 – 2 min at room temperature. Data were collected during NSRL-6c, NSRL-7a, NSRL-7c, NSRL-8a, and NSRL-8b runs. Parallel radiation geometry was used for both 2D and 3D cultures to allow the detection of foci along particle tracks (Figure 1).

Exposures with ^{137}CS γ-rays (LET = 0.91 keV/µm (Meesungnoen et al. 2001)) were carried out at the BNL Controlled Environment Radiation Facility at a dose rate of 0.5 - 1 Gy/min.

Irradiations and dosimetry were performed by the same certified operator. All γ-ray exposures were performed at room temperature with cell cultures prepared in parallel with the iron ion experiments within one day.

Immunostaining for γ-H2AX and 53BP1 in monolayers

Cells for γ-H2AX and 53BP1 immunostaining were cultured and stained in 8 well plastic chamber slides (Nalge Nunc International). After incubation at the indicated times post exposure, the cell culture medium was removed and the cells were washed twice with PBS. Cells were then fixed in 2% paraformaldehyde in PBS for 10 min at room temperature, washed twice in PBS and treated with pre-cooled 100% methanol for 30 min at -20°C to open up the plasma membrane. After two additional washes with PBS at room temperature, the slides were stored at 4°C. Wells were filled with PBS and sealed with self adhesive foil (Phenix Research Products, Hayward, CA, USA) to prevent them from drying out and then shipped to LBNL on ice. Samples were stained within four weeks after fixation. Before immunostaining the samples were washed with PBS and once with 1% bovine serum albumin (BSA, Invitrogen) in PBS, then non-specific binding was blocked by incubation for 1 h in 1% BSA/PBS at room temperature. The samples were then incubated for 1 h in a mouse monoclonal immunoglobulin (IgG) 1 anti phospho-histone H2AX (serine 139) antibody (Upstate (Millipore), Temecula, CA, USA; 1 mg/ml, 1:500 dilution in 1% BSA/PBS) and/or a rabbit polyclonal anti 53BP1 antibody (Bethyl, Montgomery, TX, USA; 1 mg/ml, 1:500 dilution in 1% BSA/PBS). After washing the cells four times in 1% BSA/1x PBS for 15 min each, they were incubated 1 h with the secondary antibody (Molecular Probes (Invitrogen); 2 mg/ml, 1:400 dilution in 1% BSA/ PBS) Alexa Fluor 488 goat anti-mouse IgG or Alexa Fluor 594 goat anti rabbit IgG and washed twice in PBS for 10 min, counterstained

with 0.1 μg/ml 4',6-diamidino-2-phenylindole (DAPI) in PBS for 5 min, and washed one more time with PBS for 10 min. Slides were air dried and mounted with Vectashield (Vector Laboratories, Burlingame, CA, USA). Picture acquisition was performed within two months. Immunostaining for γ-H2AX and 53BP1 in binucleated cells was conducted as described but used acridine orange (10 μg/ml; Sigma-Aldrich), which allowed detection of binucleated cells by cytoplasmic staining. Acridine orange staining bleached out after about 30 sec of exposure under the microscope and after that did not interfere with the γ-H2AX or 53BP1 foci which were counted in binucleated as well as mononucleated cells by eye.

Immunostaining for γ-H2AX, 53BP1 and integrin α6 in 3D acinar structures

Immunostaining of 3D samples was performed similar to monolayer staining with minor changes and the addition of integrin α6 staining to verify that the cells were morphologically polarized. After transport of the slides back to LBNL the PBS was replaced by 10% DMSO (dimethyl sulfoxide) in PBS and samples were stored at -20°C until immunostaining to prevent contamination. In addition to the BSA block cells were incubated for 1.5 h at room temperature in the undiluted blocking reagent "Mouse Detective" (Biocare Medical, Concord, CA, USA). After blocking, the cells were washed 3x in 1% BSA in 1x PBS before incubated over night at 4°C with the primary integrin α6 antibodies (Becton Dickinson, Franklin Lakes, NJ, USA; purified rat anti-human integrin α6 chain, 1:800). After washing the cells, they were incubated 1 h with the secondary antibodies (Molecular Probes (Invitrogen); Alexa Fluor 633 goat anti-rat for integrin α6 in a 1:400 dilution). Cells were counterstained with 0.5 μg/ml DAPI (Sigma-Aldrich) in 1x PBS for 5 min, covered with 1x PBS to prevent them from drying out, and kept

refrigerated until picture acquisition, which was performed within two weeks after immunostaining. Only foci in functionally polarized acini were scored (Figure 1).

Image acquisition

2D cultures were imaged using a Zeiss Axiovert epifluorescence microscope (for details see (Costes et al. 2007)) with a Zeiss plan-apochromat 40X dry lens (Carl Zeiss, Jena, Germany) and a scientific-grade 12-bit charged coupled device (CCD) camera (ORCA AG Hamamatsu, Bridgewater, NJ, USA). All images within the same data set were captured with the same exposure time so that intensities were within the 12-bit linear range and could be compared between specimens. We excluded presumptive S-phase cells identified by a high uniform label and/or a large number of small foci. Images were taken in 11 focal planes with 0.7 μm steps over a range of 7 μm total to capture foci in different focal plans. The image analysis was performed on the maximum projection of these image stacks. At least 100 and up to 600 cells per treatment group were analyzed for each independent experiment (over 100.000 cells total). Three independent experiments were performed in duplicates (N = 6) for γ -ray and iron ion exposures.

3D cultures were imaged using a Zeiss Axiovert 200M automated microscope with Ludl position-encoded scanning stage (Carl Zeiss). Images were acquired using a Zeiss plan-apochromat 63X water objective (numerical aperture (NA) of 1.2), multiband dichroic and single-band emission filters in a filterwheel, and scientific-grade EM-CCD (electron multiplying charge-coupled device) camera (Hamamatsu C9100-02, 1k by 1k pixels, , 8 x 8 μm^2 pixels). All images within the same data set were captured with the same exposure time so that intensities were within the 12-bit linear range and could be compared between specimens. A CSU-10 spinning disk confocal scanner was used to acquire optical slices of 0.75 μm thickness and

illumination was provided by 4 solid-state lasers at 405, 491, 561, and 638nm under AOTF (acousto-optic tunable filter) control. The microscope was operated under Metamorph imaging software (Molecular Devices, Sunnyvale, CA, USA). Five independent iron ion experiments, some in duplicate (N = 8), and two independent γ-ray experiments in duplicates (N = 4) were performed.

Computer-based image analysis and estimation of foci numbers

Experiments were designed and annotated with radiation quality, dosage, cell line, growth conditions, and registered with BioSig (Biological Signature) Imaging Bioinformatics platform. BioSig has been updated from its previous implementation (Parvin et al. 2002; Parvin et al. 2003) for improved data entry and analysis. Annotations of experimental parameters were then followed by registering images for each set of experimental variables (e.g., fixation time for characterizing kinetics of DNA repair) for subsequent quantitative analysis. Image analysis included nuclear segmentation using convexity (Raman et al. 2007) for 2D specimens and geometric constraints to incorporate radial organization and homogenous distribution of fluorescent signals in 3D specimens (Chang et al. 2007; Han et al. 2007; Han et al. 2010; Parvin et al. 2007). Each detected nucleus provides the context for foci analysis following maximum projection of foci on nucleus-by-nucleus basis. Some of the same computational modules are also used for foci detection through iterative radial voting (Han et al. 2007; Han et al. 2010; Parvin et al. 2007). These quantitative measurements were registered with BioSig.

Premature chromosome condensation and scoring of chromatid breaks

MCF10A were seeded in 25 cm^2 flasks (156,000 cells/flask) two days before exposure (0, 0.5, and 1 Gy) and premature chromosome condensation was induced by adding 50 nM of calyculin-A (Sigma-Aldrich) at different time points after exposure. Within the 30 min incubation time with calyculin-A detached cells were collected, centrifuged, resuspended in 0.075 M potassium chloride (KCl; Sigma-Aldrich) and incubated for 10 min at 37°C. The cells were then fixed twice in 25% glacial acetic acid (Sigma-Aldrich) in methanol (Sigma-Aldrich). Cells in fresh fixative were dropped on wet slides, air dried, and stained in 4% Giemsa (Sigma-Aldrich) for 10 min. After covering the slides with mounting media, chromatid breaks in 50 – 100 G2/M-phase cells were scored blindly. Only gaps that were wider than the width of a chromatid were counted as a chromatid break. We were unable to score chromatid breaks in the 0.3 h 1Gy iron ion sample due to weak condensation of the chromosomes and therefore the chromatid break number per Gy was calculated from the 0.3 h 0.5 Gy iron ion sample.

Least square fits and statistical analysis

Biphasic foci kinetics was modeled as a sum of two exponential decays:

$$RIF(t) = A.e^{-k_1 t} + B.e^{-k_2 t} + C \qquad 1.$$

where k_1 and k_2 are repair time constants (in hr^{-1}) for the fast and the slow components, respectively; A and B represent the fraction of radiation induced foci (RIF) resolved with fast and slow kinetics, respectively; and C is the average level of foci measured in the 0 Gy specimens. Note k_1 and k_2 are often reported as half lives ($T_{1/2} = \ln(2)/k_1$ or $T_{1/2} = \ln(2)/k_2$). Similarly to what has been done in the past to fit FAR (Fraction of Activity Released) assay data (Iliakis et al. 1990), we used a two-step procedure. Briefly, time points above 10 hours were used to determine parameters for the slow components (B and k_2) by fitting the following equation:

$RIF(t) - C = B.e^{-k_2 t}$. Then B and k_2 were substituted into equation (1) to fit A and k_1 using time points less than 10 hours. Repair constants were forced to be positive or null in the fit. In some instances (3D iron ion data for γ-H2AX and 53BP1), the two component fit was not possible (the least square procedure did not converge into unique values). In this case, data were not fitted at all, because the disappearance of foci seemed to be more complex than described by equation 1. One can estimate the proportion of the repair curve due to the fast and slow component by simply looking at the fraction of A or B over A+B. Finally, whenever RIF induction was not maximum at 20 min post-IR we excluded this time point as it would compromise accurate estimation of the fast repair component due to an incomplete RIF detection. Non linear least square fits were performed using the statistical toolbox of Matlab (The Mathworks, Inc., Natick, MA, USA). Linear fits were used for the unirradiated controls.

The values in the graphs represent the mean value ± standard error of the mean (SEM) except stated otherwise. Statistical significance between control and treated samples was calculated by using a student t-test.

Results

HMEC were grown under serum free conditions to permit controlled proliferation by adding or excluding EGF from the cell culture medium. Less than 5% of the MCF10A cells incorporated bromodeoxyuridine (BrdU) within a 24 h time window when cultured without EGF for more than 2 days (data not shown). The immortalized HMEC cell line MCF10A was used for comparing the influence of radiation quality (γ-rays versus iron ions), the cell cycle status (stationary versus cycling cells), and cell culture conditions (2D versus 3D) on foci formation, while the primary 184v finite lifespan HMEC were used to investigate the ability of cells to pass through mitosis with remaining foci.

We focused on quantitative analysis of persistent foci in HMEC growing under 2D and 3D cell culture condition several days after exposure to low and high LET radiation. Representative pictures of immunostained MCF10A cells under 2D and 3D culture conditions are shown in Figure 1. At this early time the difference in foci distribution within the cell nucleus after high and low LET irradiation is clearly evident with multiple foci forming along the iron particle tracks and a more homogeneous distribution of foci after γ-ray exposure. γ-H2AX and 53BP1 foci co-localize to a high degree at this time point as shown by the merged pictures. Under 3D cell culture conditions (Figure 1B) the cells formed acini and were polarized as seen by the integrin α6 staining located predominantly at the acinus periphery (Debnath et al. 2003; Imbalzano et al. 2009).

Quantitative foci measurements were performed using computer programs developed at LBNL (Parvin et al. 2007). An example of nuclear segmentation and detection of 53BP1 foci is shown in Figure 2. The threshold settings for the foci analysis was optimized for foci detection at later time points, since the emphasis was detection of persistent foci. This resulted in a slight

underestimation of the foci numbers at early time points due to a change in foci morphology over time with increase in foci size at later times. Changing the threshold for different time points within the same data set would have increased the initial induced number of foci but would have also added bias to the analysis.

The background of foci in non-irradiated MCF10A cells was between 2 and 5 foci per cell. This number depended on the setting for foci detection and could vary due to details of the immunostaining procedure. It is not known what these foci represent; they may or may not mark DSB (overview see (Costes et al. 2010)). However, the background was relatively constant within each experiment and it is assumed that return of the number of foci per cell after radiation to the background level of the particular experiment means that the radiation–induced foci were resolved. The primary 184v HMEC had a lower background level of 0.3 to 1 foci per cell when scored by eye, which is in line with previous observations that primary cells have a lower foci level than transformed or aging cells (Sedelnikova et al. 2004, 2008).

At early time points foci levels induced by γ-rays and 968 MeV/amu iron ions showed quite similar values, which suggests that the relative biological effectiveness (RBE) is one for foci induction. However, while initially induced RIF correlate well with DSB after exposure to γ-rays, RIF after high LET radiation reflect more DSB clusters (Costes et al. 2010). Therefore it is likely that foci numbers at early time points after high LET exposure under-represent the total number of DSB. In comparison, Sudo et al. (2008) reported RBE values for HMEC of 1.6 and 1.8 for the D_{37} (dose resulting in 37% survival) and D_{10} (dose resulting in 10% survival) respectively with about 15 – 40% of the cells surviving 2 Gy of 1 GeV/amu iron ions.

Foci formation in cycling and non-cycling 2D HMEC

We studied the formation and disappearance of γ-H2AX foci (Figure 3) and 53BP1 foci (Figure 4) after exposure to γ-rays (graphs on the left) and 968 MeV/amu iron ions (graphs on the right) in non-cycling (upper graphs) and cycling cell populations (lower graphs). Foci numbers were measured up to 48 h in cycling cells and up to 72 h in non-cycling cells respectively. Our results reveal that the disappearance of radiation induced γ-H2AX and 53BP1 foci depends both on radiation quality and proliferation status. While foci numbers for γ-H2AX (Figure 3A & 3C) and 53BP1 (Figure 4A & 4C) return to control level within 22 h after γ-ray irradiation regardless of marker or cell proliferation status, iron ion induced foci were still present at this point (Figure 3B & 3D, 4B, & 4D). However, most of persistent 53BP1 foci were removed during the next 24 h while γ-H2AX foci in non-cycling cells remained significantly higher ($p = 0.035$ for 1Gy and $p = 0.007$ for 2Gy) than control levels even 72 h after iron ion exposure (Figure 3B).

A possible explanation for the reduction of γ-H2AX foci levels over time in cells grown with EGF (Figure 3D) compared to the stationary cells (Figure 3B) would be increased apoptosis. To test this idea, apoptosis was measured in cycling and non-cycling MCF10A cells after iron ion exposure using the caspase-independent cytochrome c release assay (Goldstein et al. 2005). The level of apoptosis was very low (around 1%) in cycling and non-cycling cells (data not shown), confirming low apoptotic numbers in HMEC reported by Goldstein et al. (2005). Apoptosis therefore is unlikely to explain the loss of foci (Figure 3D).

Presence of foci in HMEC (184v) cells undergoing mitosis after irradiation

To further elucidate the mechanism of foci removal in replicating monolayer cells, we tested the ability of the cells to pass through mitosis while still carrying persistent foci (Figure 5A). Cytochalasin B and acridine orange staining were used (Figure 5B) to identify 184v HMEC that

had passed through mitosis and had formed binucleated cells. Cytochalasin B allows the cells to complete telophase and form nuclear membranes around the daughter nuclei, but prevents cytokinesis. Therefore cells can complete mitosis but not cell division.

Synchronized 184v cells were released from the cell cycle block in G1-phase by adding EGF to the cell culture medium 15 h – 21 h before exposure to 0.5 Gy iron ions, which increased the number of cells in G2-phase at the time of exposure. The addition of Cytochalasin B 1 hr after irradiation allowed cells exposed in M-phase to proceed into G1-phase and persistent foci were measured in both mononucleated and binucleated cells for comparison. As shown in Figure 5D (right), binucleated cells showed elevated γ-H2AX and 53BP1 foci numbers 3h after 0.5 Gy of iron ion exposure (2.57 ± 0.68 and 2.6 ± 0.8 respectively) compared to non-irradiated controls (0.41 ± 0.17 and 1.01 ± 0.63 respectively). This demonstrates that foci can pass mitosis. If such foci represent DSB, this process could allow further repair in the next cell cycle. The number of foci detected in the binucleated cells were similar to the foci numbers in mononucleated cells (2.03 ± 0.44 and 2.65 ± 0.98 respectively) as shown in Figure 5D (left).

γ- H2AX foci formation in HMEC after release from cell cycle block

To test the hypothesis that γ-H2AX foci induced in non-cycling cells might resolve after a later release from the cell cycle block we measured foci numbers in cells that were non-dividing during iron ion exposure and then released 22 h later (Figure 6). Cells without EGF (bold squares with solid line) showed remaining foci even 72 h after 2 Gy iron ion exposure while cells that were released 22 h after exposure were back to background level 50 h after release or 72 h after exposure respectively. The increase in foci numbers at 48 h after 2 Gy iron ion exposure for released cells (open symbols with dotted line) is most likely due to a higher number of cells in S-

phase, even though we attempted to manually exclude these cells. It can be pointed out that also the control for the released cells showed an increase at the same time even this increase is much lower compared to the exposed sample. We assume that most of the cells have already passed through S-phase in the control sample at 48h and that we see more cells in S-phase at that time in the exposed sample due to a delay in reentry of the cells in the cell cycle or slower progression through the cell cycle.

Foci formation in 3D

To determine whether tissue-specific organization affected radiation-induced foci formation and resolution, cells were seeded on top of an extracellular matrix allowing the formation of acinar structures *in vitro.* Foci formation and persistence were measured after exposure to γ-rays and iron ions (Figure 7). The number of foci for both γ-H2AX and 53BP1 had returned to control levels within 22 h of γ-ray exposure. In comparison, the number of iron-ion induced γ-H2AX foci remained significantly higher than controls for up to 48 h, while 53BP1 levels were at control levels within 22 h after exposure. Thus, the dynamics of γ-H2AX and 53BP1 foci induction and resolution for non-cycling cells were quite similar independent of cell culture configuration.

Chromatid breaks measured in premature condensed chromosomes of cycling HMEC

Measuring chromatid breaks in premature condensed chromosomes is a widely used cytogenetic method for detecting DNA damage after exposure to different qualities of ionizing radiation (for review of PCC techniques see (Gotoh and Durante 2006)). The number of chromatid breaks after γ-ray or iron ion exposure was plotted over time (Figure 8). The frequency of chromatid breaks

returned to control level by 22 h after γ-ray exposure, but remained elevated even 48 h after iron ion exposure. This result is somewhat surprising in view of our findings that γ-H2AX and 53BP1 foci in cycling cells were back to control levels within 48 h after iron ion exposure (Figure 3D & 4D). This suggests that DSB might still be present, although foci numbers are back to control levels in replicating cells

Discussion

Pulsed-field gel electrophoresis experiments have established that high LET radiation induces persistent DNA double-strand breaks for doses in the range of 25-80 Gy (Stenerlöw et al. 2000; Rydberg et al. 2005). This is interpreted to be due to the higher complexity of breaks compared to low LET radiation. In most cases, non-proliferating human fibroblasts were used in these studies and the breaks were measured up to 24 h after exposure. Much less is known about lower doses, particularly for proliferating cells at longer repair times. Karlsson and Stenerlöw (2004) found persistent γ-H2AX foci for up to 24 h after 1 Gy of high LET nitrogen-ion irradiation in non-proliferating normal human fibroblasts. Takahashi et al. (2008) showed a slower decrease in the γ-H2AX intensity using flow cytometry after exposure to 500 MeV/amu iron ions compared to X-ray irradiation in exponentially growing human AG01522 fibroblasts and Asaithamby et al. (2008) showed similar results in γ-H2AX immunostained human HSF42 skin fibroblasts 24 h after iron ion exposure (300 MeV/amu and 1 GeV/amu). Desai et al. (2005) also showed a LET dependency for the disappearance of γ-H2AX clusters in confluent normal human fibroblasts between iron (176 kev/μm) and silicon (54 keV/μm) ions. While both radiation qualities induced persisting γ-H2AX foci 24 h after exposure, cells exposed to iron ions showed significantly higher residual foci numbers compared to silicon ions. In each of these studies (Desai et al. 2005; Asaithamby et al. 2008; Takahashi et al. 2008), γ-H2AX foci were measured up to 24 h after exposure.

In the present study we measured foci frequency for up to 72 h and compared the response in non-proliferating and proliferating HMEC. We found that a fraction of γ-H2AX foci persisted for at least 72 h after high LET iron ion radiation in non-proliferating cells, in contrast to foci

induced by γ-rays that disappeared after 22 h. This confirms the earlier studies. We and others have interpreted these persistent γ-H2AX foci as evidence of persistent DSB. However the situation was different for 53BP1 foci, which constitute another marker of DSB. These foci returned to background level by 48 h. One can hypothesize that the absence of 53BP1 binding indicates lack of repair at these late time points. Another interpretation is that 53BP1, which is recruited to open chromatin, is excluded when a DSB is rejoined, but γ-H2AX, which is a phosphorylation that requires a phosphatase to resolve, is decoupled from the process. It can be pointed out that Karlsson and Stenerlöw (2004) in the above mentioned study found that another repair protein, MRE11 (meiotic recombination 11 homolog), showed less persistence of foci compared to γ-H2AX. Thus, persistent foci that lack essential repair factors, such as 53BP1 and MRE11, could be indicative of lack of repair and persistence of DSB, or the absence of 53BP1 and MRE11 could be indicative of completed repair, and γ-H2AX marks something different. However, in contrast to the situation in non-proliferating cells, all foci were removed in proliferating cells within 48 h after iron ion exposure. This suggests that necessary repair pathways for complex DNA damage, such as homologous recombination or backup pathways of non-homologous end joining (B-NHEJ) (Iliakis 2009), might be needed but are not available in non-proliferating G1 cells. Another possibility is that chromatin reorganization or other processes during DNA replication or mitosis facilitated DSB repair and/or γ-H2AX dephosphorylation. Various reports support these hypotheses. Frankenberg-Schwager et al. (2009) are suggesting an important role of homologous recombination (HR) and single-strand annealing (SSA) for complex DSB in S-phase after studying DSB repair in the Chinese hamster ovary (CHO) cell line AA8 and its repair deficient derivatives after X-ray or α-particle exposure. A role for homologous recombination in removing a fraction of clustered lesions induced by iron

ion radiation was recently demonstrated (Zafar et al. 2010), and B-NHEJ has been shown to be compromised in plateau-phase cells and be most active in G2 (Iliakis 2009). Kato et al. (2008) studied the induction and disappearance of DNA DSB in synchronized CHO cells after γ-ray exposure, comparing γ-H2AX foci numbers and DSB values measured with the gel electrophoresis assay. Their data showed much slower disappearance of γ-H2AX foci in mitotic than G1-phase cells while no difference was seen for the gel electrophoresis assay. The authors suggest that the limited accessibility of dephosphorylation enzyme in metaphase cells or trapped γ-H2AX in condensed chromatin is responsible for the slower dephosphorylation. Increase of accessibility and decondensation of the chromatin during S-phase could also explain our observation of γ-H2AX foci removal in cycling HMEC. However we found that mitosis per se did not make foci disappear at short times after irradiation, but instead foci were inherited by the daughter cells. This experiment was performed using primary 184v cells at early times after irradiation, but we expect this is also true for the immortal cell line MCF10A used for all other experiments which can be expected to have less strict cell cycle control mechanisms. However, since our experiments were performed at early time points (1-3 hr after irradiation) we cannot exclude the possibility that persistent foci were removed as a consequence of chromatin reorganization at mitosis at later time points.

To discriminate between DSB repair and γ-H2AX dephosphorylation we monitored chromatid breaks in prematurely condensed chromosomes in proliferating cells at 48 h after irradiation when the foci had disappeared. We found that an excess of chromatid breaks were still present in iron-irradiated cells but not in γ-irradiated cells. Although the precise relationship between DSB and chromatid breaks are not known (Bryant et al. 2004, 2008), this suggests that persistent DSB were still present even though the foci were absent. A possibility is that the breaks at that time

were permanent and no longer candidates for repair. Overall, our studies suggest a more complex picture for DSB repair and foci formation and removal than previously anticipated, and suggest an uncoupling of DSB and foci at later repair times after high LET radiation. In other studies (Suzuki et al. 2006; Kato et al. 2008) it has been suggested that foci may be present but not always mark DSB, for example in senescent cells, while our data comparing chromatid breaks and foci suggest that the opposite situation may also occur, that DSB are present but not marked as foci.

We also measured radiation induced γ-H2AX and 53BP1 foci formation and their disappearance in HMEC grown under either 2D or 3D culture conditions. As far as we know, this is the first published study of foci formation and removal in HMEC grown under 3D conditions. Roig et al. measured colocalized foci of DNA-PKcs (DNA-dependent protein kinase, catalytic subunit) and γ-H2AX in human colon epithelial cells in 2D and 3D after 1 Gy of 1 GeV/u protons or iron ions and came to the conclusion that the kinetics of DNA damage and repair in epithelial cells after exposure to low- or high-LET radiation is similar in the 2D and 3D environment (Roig et al. 2009). The authors report that 20% - 30% of the induced colocalized foci are still remaining 24 h after iron ion exposure in 2D and 3D cultures. These data fit well with our results. We still see about 20% remaining γ-H2AX foci in cycling cells 22 h after exposure in 2D (Figure 3D) as well as in the 3D cultures (Figure 7B) but the remaining γ-H2AX foci in 2D are resolved within the next 24 h and foci levels are back to baseline 48 h after iron ion exposure (Figure 3D). Roig et al. measured foci removal only up to 24 h; therefore it is not known if the persisting foci at 24 h in colon epithelial cells would have been resolved 48h after iron ion exposure.

Similar to Roig et al. we found that the general pattern of foci formation and removal at late time points were the same in both conditions. In particular, the number of remaining γ-H2AX and

53BP1 foci at 48 h was similar. However, we noted a slightly faster 53BP1 foci removal at early time-points in 3D cultures after γ-ray exposure ($T_{1/2}$ (fast) for 2D was 3.5h compared to 2h for 3D) while a similar removal was observed for γ-H2AX in non-cycling cells. Interestingly, a two component fit could not adequately describe the apparently more complex foci removal in 3D after iron ion exposure, so a direct comparison of $T_{1/2}$ for foci removal at early times between the 2D and 3D data in non-cycling cells after iron ion exposure was not possible. The significance of this observation is not known at present. These results at low doses and long repair times in proliferating and non-proliferating HMEC cells support the notion that persistent damage is induced by high LET radiation. This in turn might be a factor that contributes to the higher efficiency of high LET radiation compared to low LET radiation for a variety of radiation effects occurring later.

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Figure legend

Figure 1: Immunostaining of radiation induced γ-H2AX (second column) or 53BP1 (third column) foci and colocalization of both (fourth column) in MCF10A cells grown as a 2D monolayer (A) or as 3D acini (B) 0.3 h (2D) or 1 h (3D) after exposure of 2 Gy of γ-rays (second row) or iron ions (third row). First row shows unirradiated controls. Counterstaining was with DAPI (first column). To test for functional polarization, acinar structures were stained in addition for α6-integrin (fifth column in B). Only functionally polarized acini were included in the analysis.

Figure 2: Example of a computer based analysis of radiation induced 53BP1 foci in HMEC (MCF10A) 0.3 h after 2 Gy of iron ion exposure. Detected nuclei are circled in red and foci detected by the analysis are circled in blue in the right panel.

Figure 3: Induction and repair of radiation induced γ-H2AX foci in non-cycling (A & B) and cycling (C & D) MCF10A cells after γ-ray (A & C) or iron ion (B & D) exposure. The mean numbers of foci per cell are plotted over time. Remaining γ-H2AX foci can be observed in non-cycling cells up to 72 h after 1Gy ($p = 0.035$) or 2Gy ($p = 0.007$) iron ion exposure (B). In cycling cells foci levels are back to control after γ-ray (C) or iron ion (D) exposures within 22 h or 48 h respectively. No significant difference in the dynamic of γ-H2AX dephosphorylation is visible in cycling or non-cycling cells after γ-ray exposure (A & C). Error bars indicate the

standard error of the mean (SEM) for N = 6 (three independent experiments in duplicates). The curves represent two component exponential least square fits.

Figure 4: Induction and repair of radiation induced 53BP1 foci in non-cycling (A & B) and cycling (C & D) MCF10A cells after γ-ray (A & C) or iron ion (B & D) exposure. The mean numbers of foci per cell are plotted over time. Foci levels in HMEC are back to control levels within 22 h after γ-ray exposure (A & C) or 48 hrs after iron ion exposure (B &D) in both non-cycling and cycling cells. Error bars indicate the standard error of the mean (SEM) for N = 6 (three independent experiments in duplicates). The curves represent two component exponential least square fits.

Figure 5: Panels A to C: Measuring the inheritance of foci in binucleated cells. Synchronized HMEC (184v) were released from a temporary cell cycle block in G1-phase 15 – 21 h before exposure and then treated with cytochalasin B as indicated in panel A. Binucleated cells were identified by acridine orange staining (B). An example of 53BP1 foci in mono- and binucleated HMEC is shown at 3h after iron ion exposure in panel C. Panel D: Measurement of the presence of radiation induced foci in mononucleated (left) and binucleated (right) HMEC (184v) 3h after 0.5 Gy of iron ion exposure. Mean numbers of foci per mononucleated (left) or binucleated (right) cell are plotted for γ-H2AX or 53BP1. The binucleated cells have divided once after irradiation and still show elevated foci numbers for γ-H2AX ($p = 0.0009$) and 53BP1 ($p = 0.031$) compared to the control levels. This indicates that cells carrying foci and consequently DNA damage are able to pass through mitosis and produce progenitors. Error bars indicate the standard deviation for N = 3 (experiment in triplicates).

Figure 6: Induction and repair of radiation induced γ-H2AX foci in non-cycling (filled symbols with bold line) MCF10A cells that were released from a cell cycle block by adding EGF to the cell culture medium 22 h after iron ion exposure (open symbols with dotted lines). Remaining γ-H2AX foci can only be observed in non-cycling cells while foci levels in released cells returned to control level within 50 h after the release (72 h after exposure). The increase in foci numbers at 48 h in the released cells (dotted line) is most likely due to a higher number of S-phase cells at that time. Error bars indicate standard error of the mean (SEM) for N = 6 (two independent experiments in triplicates).

Figure 7: Induction and repair of radiation induced γ-H2AX (A & B) and 53BP1 (C & D) foci in non-cycling MCF10A cells cultured in 3D after 2Gy of γ-ray (A & C) or iron ion (B & D) exposure. The mean numbers of foci per cell are plotted over time. Remaining γ-H2AX foci can be observed in cells up to 48h ($p = 0.002$) after iron ion exposure (B) while 53BP1 foci are back to control levels (D). Error bars indicate the standard error of the mean (SEM) for N = 4 (γ-ray; two independent experiments in duplicates) or N = 8 (iron ion; five independent experiments, three in duplicate). The γ-ray curves represent two component exponential least square fits. The iron ion data could not be fitted by a two component exponential curve (see materials and methods for details).

Figure 8: Induction and repair of radiation-induced chromatid breaks (ctbs) in prematurely condensed chromosomes (PCC) of cycling MCF10A cells after 1 Gy of γ-ray (triangles) or iron

ion (squares) exposure. The mean numbers of chromatid breaks per cell are plotted over time. While the number of chromatid breaks returns to control level (circles) within 22 h after γ-ray exposure we detect remaining chromatid breaks even 48 h after iron ion exposure. The iron ion 1 Gy 0.3 h data point was extrapolated from a 0.5 Gy 0.3 h data point (6.26 ± 0.71) due to insufficient chromosome condensation of the 1 Gy 0.3 h sample. Error bars indicate standard error of the mean (SEM) of the number of chromatid breaks between cells of the same treatment group (single experiment). At least 50 cells were scored for each data point.

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