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Two stages of XRCC1 recruitment and two classes of XRCC1 foci formed in response to low level DNA damage induced by visible light, or stress triggered by heat shock

Kamil J. Solarczyk, Magdalena Kordon, Krzysztof Berniak, Jurek W. Dobrucki*

Division of Cell Biophysics Faculty of Biochemistry, Biophysics and Biotechnology, Jagiellonian University ul. Gronostajowa 7, 30-387 Kraków, Poland

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ABSTRACT

Induction of local photosensitised DNA damage has been used to study recruitment of repair factors, spatial organisation and subsequent stages of the repair processes. However, the damage induced by a focused laser beam interacting with a photosensitiser may not fully reflect the types of damage and repair encountered in cells of an animal under typical conditions in vivo. We report on two characteristic stages of recruitment of XRCC1 (a protein engaged in BER and SSB repair pathways), in response to low level DNA damage induced by visible light. We demonstrate that, when just a few DNA breaks are induced in a small region of the nucleus, the recruited XRCC1 is initially distributed uniformly throughout this region, and rearranges into several small stationary foci within minutes. In contrast, when heavy damage of various types (including oxidative damage) is induced in cells pre-sensitized with a DNA-binding drug ethidium bromide, XRCC1 is also recruited but fails to rearrange from the stage of the uniform distribution to the stage of several small foci, indicating that this heavy damage interferes with the progress and completion of the repair processes. We hypothesize that that first stage may reflect recruitment of XRCC1 to poly(ADP-ribose) moieties in the region surrounding the single-strand break, while the second-binding directly to the DNA lesions. We also show that moderate damage or stress induces formation of two types of XRCC1-containing foci differing in their mobility. A large subset of DNA damage-induced XRCC1 foci is associated with a major component of PML nuclear bodies - the Sp100 protein.

1. Introduction

In recent years, introduction of several techniques of inducing DNA damage, which is confined to a small region of the cell nucleus, has boosted the field of DNA repair [1–4]. A number of proteins have been shown to be recruited to locally inflicted DNA damage, and their kinetics and interactions have been studied extensively. However, some concern has been voiced regarding the relevance of the local lesions, which are usually induced by a combination of focused laser light and exogenous photosensitisers, with respect to naturally occurring DNA damage. The main reservations stem from the fact that laser-based techniques often lead to massive DNA damage and activation of multiple DNA repair pathways [5,6].

A variety of techniques or agents, capable of inducing DNA damage distributed randomly throughout the whole nucleus, have also been used [7]. DNA lesions induced in many locations within the whole nuclear volume are usually manifested by formation of so-called Radiation Induced Foci (RIF) [7–9]. These foci, representing local accumulation of repair proteins, are thought to be formed at the sites of double- as well as single-strand DNA breaks [10].

Interestingly, the small foci rich in repair factors, of the type that are formed in response to ionizing radiation affecting the whole cell, are usually not observed after laser-induced local damage. In the majority of published microscopic images, repair proteins recruited to the damaged region (as well as phosphorylated histone H2AX [5,11–15]) are evenly distributed and remain there in a form of a diffused, cloud-like region [1,11,12]. The cause of this difference between spatial patterns of DNA repair proteins and markers in IR-like and laser-induced damage is unclear, but it is plausible that the density of the induced lesions is the major factor involved. Immediate induction of hundreds of double-strand breaks, and possibly

Abbreviations: BER, base excision repair; DSB, double-strand DNA break; IF, immunofluorescence; mJ, milliJoul; MMS, methyl methane sulfonate; NB, nuclear (subnuclear) body; PAR, poly(ADP-ribose); PML, promyelocytic leukemia; RIF, radiation-induced foci; SSB, single-strand DNA breaks; RFP, red fluorescent protein; XRCC1, X-ray repair cross-complementing protein 1.

* Corresponding author. Fax: +48 12 664 55 03.
E-mail address: jerzy.dobrucki@uj.edu.pl (J.W. Dobrucki).

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even more lesions of other types (including photo-oxidised bases and single-strand breaks) by a focused laser beam, all within a small nuclear volume, is practical as it facilitates microscopy detection of the damage site. However, induction of such an extensive damage leading to accumulation of multiple copies of fluorescently tagged DNA repair proteins raises doubts regarding the ability of cells to repair this damage, and exposes the experimenter to a risk of missing important clues concerning spatial organization of the DNA damage response.

One of the best examples of the influence of damaging conditions on spatial organisation of DNA repair processes is provided by recruitment of XRCC1, a protein engaged in BER and SSB repair pathways [16–24]. Rapid recruitment of XRCC1 to locally induced DNA damage has been demonstrated in many studies [19,25–27]. XRCC1 begins to accumulate at the damage site within seconds, and reaches its maximum concentration within minutes after induction of local damage [19]. Similarly to other DNA repair factors, XRCC1 tagged with a fluorescent protein usually forms a cloud-like pattern at the site of local laser-induced damage [19,26,28]. There is evidence, however, that this protein can also form a pattern of foci in response to local DNA damage. XRCC1 foci have been observed after treatment with various agents - MMS[26], H_2O_2 [29], $KBrO_3$[27], ionizing radiation [10] or even after siDNA introduced to cells to mimic single-strand breaks [30]. Recently, it has been shown that XRCC1 can form foci after illumination with a low intensity 405 nm light [31] (in the absence of exogenous photosensitisers), as well as in the vicinity of regions illuminated with a UV-laser [32]. All these observations, combined with the fact that some XRCC1 foci can also be found in nuclei of cells grown under physiological conditions, indicate that accumulation of multiple copies of XRCC1 in a small focus in the nucleus is functionally important, but the architecture and role of these structures, and the influence of damaging conditions on their formation, remain unclear. The goal of this work was to image recruitment of XRCC1 to localized, low level DNA damage induced by visible light in order to provide new insights into the spatial patterns formed by the accumulated protein within the damage site. We demonstrate that initially the concentration of XRCC1 is uniform throughout the previously illuminated region, within minutes, however, XRCC1 is drawn into several small stationary foci. In contrast, when heavy damage of various types (including oxidative damage) is induced in cells presensitized with ethidium bromide, XRCC1 is also recruited but fails to rearrange from the stage of the uniform distribution to the stage of several small foci, indicating that this heavy damage interferes with the progress and completion of the repair processes. Highly mobile as well as almost immobile foci are formed in response to DNA damage induced by visible light or stress triggered by heat shock. A subset of XRCC1 foci is associated with a major component of PML nuclear bodies - the Sp100 protein.

2. Materials and methods

2.1. Cell culture and transfection

HeLa 21-4 cells were maintained in DMEM (Sigma–Aldrich, D5523) supplemented with 10% fetal bovine serum (FBS) (Gibco, UK, 10,106–169) at 37 °C and 5% CO_2. Cells were seeded on 18 mm–diameter coverslips (Menzel–Glaser; Braunschweig, Germany) and after 24 h either imaged in DMEM/F12 (Sigma–Aldrich, Poznań, Poland) supplemented with 2% FBS, or transfected with RFP-XRCC1 and/or GFP-Sp100A plasmid [33] using FuGene 6 transfection reagent (Roche; Basel, Switzerland), according to the manufacturer's instructions, cultured for another 24 h and then imaged.

2.1. Immunofluorescence

For the visualisation of PAR and XRCC1, cells were locally damaged using blue light and then incubated under standard conditions (1 and 5 min, respectively) in order to allow the formation of PAR polymers and the recruitment of XRCC1 protein. Then the cells were fixed with 4% formaldehyde (15 min; EMS, Hatfield, PA), permeabilised (10 min) with 0.1% Triton X-100 (Sigma, Poznań, Poland), blocked (30–60 min) in 3% BSA (Sigma, Poznań, Poland) and incubated with primary (1 h) and secondary antibodies (2 h). Antibody dilutions were prepared in 3% BSA in PBS, washes were done with PBS. All procedures were carried out at room temperature. Primary antibodies were: mouse anti-XRCC1 (ab1838; Abcam, Cambridge, UK) and mouse anti-PAR (clone 10H, Enzo Life Sciences). The secondary antibody used was goat anti-mouse Alexa Fluor 488 (A11001, Invitrogen, Carlsbad, CA).

2.2. DNA damage induction

Local DNA damage was induced in cells maintained under optimal growth conditions on the stage of Leica TCS SP5 II confocal microscope as described before [34]. Briefly, a region of a nucleus of 2.5 × 2.5 μm was scanned several times (using the FRAP Wizard implemented in LAS AF; Leica Microsystems, Wetzlar, Germany) with 488 nm laser beam (in the absence of exogenous photosensitisers) at a resolution of 512 × 512 pixels and at a scanning speed of 200 lines per second. The total dose of energy delivered by the laser beam to the illuminated region (2.5 × 2.5 μm in the confocal plane when local damage was induced) was 3, 5 or 12 mJ (0.48, 0.8 or 1.92 mJ/μm^2). Note that, as in the case of ionising radiation, only a small fraction of this energy is absorbed by cellular components. For induction of local DNA damage in the presence of a photosensitiser, cells were incubated with ethidium bromide (400 nM) for 20 min, then rinsed, and a selected nucleus was illuminated with green laser light (514 nm). The total dose delivered to the illuminated region was 30 mJ (4.8 mJ/μm^2).

In order to induce stress throughout the nucleus, cells were subjected to visible (blue or green) light, or treated with heat shock. A 31 × 31 μm region was repeatedly scanned with blue (488 nm) or green (543 nm) light at a resolution of 512 × 512 pixels, at a scanning speed of 400 lines per second. The total dose delivered during this illumination was 20 mJ (0.02 mJ/μm^2). In order to induce heat shock a temperature of the medium was raised to 42 °C for 30 min.

2.3. Confocal imaging

Images and movies were acquired using Leica TCS SP5 II confocal microscope equipped with a 63x NA 1.4 oil immersion lens. For live cell experiments the cultures were maintained at 37 °C in DMEM/F12, without Phenol Red, buffered for air (pH 7.0) and supplemented with 2% FBS. Fixed cells were imaged in PBS. Images were acquired at 512 × 512 or 1024 × 1024 (8-bit) resolution with up to 3 frames averaged. For GFP and Alexa Fluor 488 excitation the 488 nm line (argon laser) was used and fluorescence emission was collected in the range 510–610 nm. RFP was excited with 561 nm laser line and the emission was collected in the range 570–750 nm. For images of cells with both GFP and RFP present, excitation was performed sequentially, using 488 and 561 nm laser lines, while the emissions were collected at 500–530 and 660–750 nm bands, respectively. The light intensities used for live cell imaging were in the range 0.24–0.35 mW (i.e. one order of magnitude weaker than the laser beam used for inducing local damage), and the doses of energy delivered to the whole field of view, while collecting data for one image, were in the range of 0.6–0.9 mJ (i.e. the doses deliv-