In Situ Visualization of DNA Double-Strand Break Repair in Human Fibroblasts


A method was developed to examine DNA repair within the intact cell. Ultrasoft x-rays were used to induce DNA double-strand breaks (DSBs) in defined subnuclear volumes of human fibroblasts and DNA repair was visualized at those sites. The DSBs remained in a fixed position during the initial stages of DNA repair, and the DSB repair protein hMre11 migrated to the sites of damage within 30 minutes. In contrast, hRad51, a human RecA homolog, did not localize at sites of DNA damage, a finding consistent with the distinct roles of these proteins in DNA repair.

Proteins that mediate certain aspects of DNA metabolism, such as DNA replication, appear to be compartmentalized within the nucleus. DNA replication therefore requires the movement of DNA to and from established sites within the nuclear matrix. Cytologic analyses have revealed that the DSB repair proteins hRad51 and the hMre11-hRad50 complex assemble in discrete nuclear foci as part of the normal cellular response to DNA damage (2-5). These findings may indicate that DNA repair does not entail the movement of DNA DSBs to preexisting intranuclear sites. Rather, they suggest that DNA repair proteins move to sites of DNA damage. The inability to detect DSBs in situ has made it difficult to address this issue experimentally. A method to induce and subsequently detect DSBs within a defined subnuclear volume would, in principle, provide a means to determine whether DSB repair requires the movement of DNA repair proteins to the sites of DNA damage.

To that end, we developed a method to examine the temporal and spatial nature of DSB repair within the context of the intact cell. This method relies on synchrotron-generated ultrasoft x-rays (<5000 electron volts (5 keV)), a multilayer monochromator for tunable ultrasoft x-ray energies with sufficient intensity for irradiation of live human fibroblasts (6), and microfabricated irradiation masks to induce DNA damage in discrete subnuclear regions of irradiated cells (Fig. 1) (7). The irradiation masks were fabricated with x-ray lithography and consist of gold stripes (1.35 µm wide with 1.35-µm separation) deposited on thin SiN membranes (7). Positrons with the irradiation mask showed that gold-shielded regions receive about 0.5% of the dose absorbed by the nonshielded regions (7). Irradiated cells thus absorb ultrasoft x-rays in 1.35-µm-wide stripes separated by 1.85-µm gaps that remain essentially unirradiated.

The 1.34-keV ultrasoft x-rays used in these experiments act almost exclusively through photoelectric interactions in biological material (8), resulting in low-energy electrons that have very short track lengths (<50 nm), comparable to the dimensions of biologically relevant structures such as chromatin (9). These properties suggested that photoelectrons and Auger electrons as well as free radicals resulting from absorption of ultrasoft x-rays would induce DNA damage almost exclusively within the 1.35-µm stripes imposed by the grids. Human fibroblasts (37Lu) were irradiated and DSBs were labeled with bromodeoxyuridine triphosphate (BrDu) and terminal deoxynucleotidyltransferase (TdT) for visualization with fluorescein isothiocyanate (FITC)-conjugated monoclonal antibody to BrDu (FITC-anti-BrDu) 30, 90, or 300 min later (10-12). Under the conditions used, TdT does not label single-strand DNA nicks (12). Nuclei observed 30 min after irradiation displayed a strong FITC signal in parallel stripes corresponding to BrDu incorporation at DNA ends (Fig. 2A). Each pair of unirradiated-irradiated stripes is 3.2 µm wide (1.85 µm unirradiated plus 1.35 µm irradiated). Hence, most nuclei (average diameter 15 to 20 µm) contained six or seven FITC-staining stripes (Fig. 2A and B). Confocal microscopy demonstrated that parallel stripes of BrDu incorporation were uniform through-
out the volume of the nucleus at this time point (Fig. 2, F to I) (13). These data indicate that ultrasoft x-rays induce highly localized DNA damage in living cells.

The FITC signal corresponding to BrdU-labeled DSBs disappeared between 90 (Fig. 2, C and D) and 300 min (14) after irradiation. Single cell electrophoresis (comet) assays revealed that the failure to detect FITC stripes at later time points was due to repair rather than redistribution of the DSBs (15). Similar DSB repair kinetics were previously observed after ultrasoft x-irradiation without an irradiation mask (16).

We reasoned that if DSBs were largely stationary before repair, their persistence in a DNA repair-deficient cell would lead to the persistence of DSB stripes. To test this hypothesis, we stripe-irradiated the DSB repair-deficient human fibroblast cell line 180BR (17), BrdU-labeled DNA DSBs with TdT, and stained the cells with FITC-anti-BrdU to visualize DSBs. In contrast to 37Lu cells, in which BrdU incorporation was undetectable by 90 min postirradiation, the 180BR cells had readily observable FITC-anti-BrdU stripes as late as 300 min postirradiation (Fig. 2E). Previous analyses had shown that minimal DSB repair occurs over this time course in 180BR cells (2, 17). These data indicate that DSBs are held in a relatively fixed position, at least in the early stages of DNA repair, and suggest that the bulk of DSB repair does not involve movement of DNA lesions through intranuclear space. In this regard, DSB repair differs from DNA replication, which is mediated by compartmentalized nuclear proteins (1). Instead, the spatial behavior of DSBs suggested that DSB repair requires the recruitment of DSB repair proteins to sites of damaged DNA.

To test this hypothesis, we examined whether two DSB repair complexes, one that includes hMre11 and hRad50 and another that includes hRad51, were recruited to the site of DSBs. Several criteria indicate that these protein complexes, both of which form nuclear foci in response to the induction of DSBs (2, 3), play distinct roles in DSB repair. The sequence of the hMre11-hRad50 complex is similar to the bacterial exonuclease SbcCD sequence (18), which suggests that this complex functions as an exonuclease that may be involved in the processing of DNA ends before strand exchange and religation (19–21). Because hRad51, a human RecA homolog, mediates DNA strand exchange (22), its action is likely to be required later than that of the hMre11-hRad50 protein complex in DSB repair. Null Scmre11 and Scra51 mutations in Saccharomyces cerevisiae indicate that the yeast Mre11-Rad50-Xrs2 complex, but not ScRad51, plays an important role in non-homologous end joining in this organism (19, 21). Finally, both biochemical and cytologic experiments indicate that hRad51 is not physically associated with the hMre11-hRad50 complex (2).

To determine whether partial volume irradiation could distinguish the actions of hRad51 and hMre11 in intact nuclei, we doubly stained cells for DSBs (FITC-anti-BrdU) and hMre11 (anti-hMre11) (23). Analysis of stripe-irradiated doubly stained 37Lu cells revealed that, as early as 30 min after irradiation, the BrdU-labeled DSBs and hMre11 protein colocalized (Fig. 3). The abundance and composition of the hMre11-hRad50 complex are identical in irradiated and unirradiated cells (2, 24). Hence, the association of these proteins with damaged DNA was due to migration of existing hMre11-hRad50 complexes. Optical sectioning of hMre11 stripe-positive nuclei revealed that hMre11 protein was localized in stripes throughout the nuclear volume (14). The FITC signal (corresponding to DSBs) disappeared by 90 to 300 min after irradiation, whereas hMre11 staining became diffuse, as it is in unirradiated cells.

Vestigial hMre11 stripes persisted in about 5% of nuclei examined at 300 min (Fig. 4C). Previous studies of DSB repair in human cells have indicated that the bulk of ionizing radiation-induced DSBs are repaired within the first 60 min after irradiation and that a more slowly repaired population of DSBs persists for 24 to 48 hours (16, 25). Our data suggest that the hMre11-hRad50 complex functions in the fast component of DSB repair. Because we previous-

![Fig. 2. Partial volume irradiation of 37Lu or 180BR human fibroblasts (7). Cells were labeled for DSBs with BrdU and FITC-anti-BrdU (A, C, and E), followed by DAPI (B and D) counterstaining (10). (A and B) 37Lu, 30 min after irradiation; bar, 5 μm; (C and D) 37Lu, 90 min after irradiation; bar, 5 μm; (E) 180BR, 5 hours after irradiation; bar, 5 μm; (F to I) optical sections of a partially irradiated 37Lu nucleus labeled for DSBs with BrdU (10). Representative images separated by 1.0 μm are shown (F), with increasing distance from the x-ray beam from (F) to (I). The high background observed in (F) is caused by autofluorescence from the Mylar plating surface.](image)

![Fig. 3. Colocalization of BrdU incorporation and hMre11 stripes. Thirty minutes after irradiation, 37Lu fibroblasts were sequentially labeled (23) to detect BrdU (FITC-anti-BrdU) (A), hMre11 (B), and DNA (DAPI) (C). (D) Merged image of (A) to (C). Bar, 10 μm.](image)

![Fig. 4. Localization of hMre11 to stripes after partial volume irradiation. 37Lu (A to D) or 180BR (E) fibroblasts were fixed after partial volume irradiation and stained for hMre11 or hRad51 (26). (A) Anti-hMre11, 30 min after irradiation; (B) anti-hRad51, 30 min after irradiation; (C) anti-hMre11, 5 hours after irradiation; note that one nucleus (arrow) shows a weak stripe pattern; (D) anti-hRad51, 5 hours after irradiation; (E) anti-hMre11 in 180BR cells, 5 hours after irradiation. Bars in (A) and (D), 10 μm; bars in (B), (C), and (E), 5 μm.](image)
ly showed that these proteins also function in the slower component of DSB repair (2), the residual hMre11 stripes may indicate that the number of slowly repaired DSBs falls below the limit of TdT detection, whereas immunofluorescent detection of hMre11 is sufficiently sensitive to identify these slower repair events.

To determine whether localization of hMre11 was attributable to the presence of DSBs, we examined whether persistent hMre11 was attributable to the presence of these slower repair events.

The residual hMre11 stripes may indicate the presence of DNA damage (31). Based on the failure to suppress DNA synthesis after ionizing radiation of cells established from ataxia telangiectasia patients, a similar function has been proposed for ATM (32). The use of mutants such as dμ-pk and ATM in partial volume irradiation assays may help to define defects in signaling functions required for relocalization of DSBR repair proteins.

REFERENCES AND NOTES

10. Logarithmic-phase human fibroblasts plated on 8-μm Mylar were irradiated (7) with a mean nuclear dose of 100 Gray. The dose was calculated by using the known entrance dose (D0), the attenuation coefficient (μ) of 1.34 keV·cm (0.2 mm−1), and the estimated cell thickness (Tc = nuclear thickness (5 μm), Tc = cytoplasmic thickness (0.5 μm)) in the equation D = D0 μ(Tc) = D0 X/μ + D0 (1−X/μ) (where X = 0.15). Cells were fixed and permeabilized 30 min, 90 min, or 5 hours after irradiation as described in (12). (Technical limitations precluded analysis of cells irradiated earlier than 5 min after X-irradiation.) Cells were briefly washed in double-distilled H2O and incubated for 60 min at 37°C in labeling mixture [100 mM sodium cacodylate (pH 7.6), 1 mM CoCl2, 0.2 mM dithiothreitol, 0.00001% Triton X-100, 45 nM of FITC-conjugated antibody to rabbit immunoglobulin (Jackson ImmunoResearch, West Grove, PA) was substituted for FITC-conjugated secondary antibody N-terminal dephosphorylation with a cooled charge-coupled device camera and images were processed using Photoshop 4.0 (Adobe).
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