Markers of the Low-Dose Radiation Response

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Abstract

The goal of this work is to develop a technology that will allow direct visualization of DNA double-strand break repair complexes *in situ* in the nuclei of irradiated cells.

Background: Ionizing radiation has a unique ability to induce damage simultaneously at multiple sites within a spatially restricted region of DNA. The resulting double-strand DNA breaks (DSBs) present a major threat to the integrity and stability of the genome. Our understanding of the origin and fate of DSBs is based primarily on studies at high radiation doses. Despite recent progress toward the elucidation of molecular mechanisms underlying DSB repair, technologies are still not available to visualize individual DSBs and DSB repair complexes *in situ*, in irradiated cells.

Specific Aims: This project involves development of a technology to allow direct *in situ* visualization of DSB repair complexes. These studies will help bridge the gap between biochemical studies of repair enzymes and an understanding of the process of repair as it actually occurs within the radiation-injured cell. Specific aims include:

- The **development of tools** for *in situ* visualization of DSB repair complexes.
- The **use of these tools** to detect and characterize visible subnuclear structures associated with DSB repair.
- A demonstration of the **practical utility** of the tools and assays developed in Aims 1 and 2 by quantitative studies of DSB repair.

Project Organization

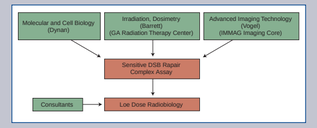
This project involves the collaboration of specialists in several areas:

A molecular and cell biology group, working directly with William Dynan (Principal Investigator), will generate stable cell lines expressing fluorescently tagged repair proteins, develop antibodies to repair proteins, and use these tools to visualize radiation-induced changes in protein localization.

An experienced radiobiologist, John Barrett (co-Principal Investigator), will oversee irradiation and dosimetry using the facilities of the Georgia Radiation Therapy Center.

An imaging technology specialist, Steven Vogel (co-Principal Investigator), will apply state-of-the-art technology, including multiphoton microscopy, for *in situ* visualization of repair complexes.

Consultants from the Savannah River Ecology Laboratory (a unit of the University of Georgia based at the US DOE Savannah River site) will provide advice on the design and interpretation of low dose radiation experiments.



Relevance to Environmental Management Needs

The critical lesions induced by ionizing radiation are thought to be DNA double-strand breaks. The main objectives of the proposed work are to develop a set of reagents that will allow visualization of double-strand break repair complexes within individual cells and to use these reagents to perform quantitative studies of the biological response to low-dose radiation. This is an interdisciplinary effort that will bring together specialists in molecular biology, radiobiology, and imaging technology.

Our hope is that this new assay for double-strand break repair will be two orders of magnitude more sensitive than existing physical methods of measuring double-strand DNA breaks. This increase in sensitivity means that the assay will be applicable to studies conducted at very low doses and low dose rates. The new technology will facilitate quantitative studies of the origin and fate of the small numbers of double-strand breaks that are believed to underlie the effects of radiation at low doses. It will allow the response to low-level injury to be measured directly, eliminating the need to rely on extrapolation from measurements made at higher doses.

The development of a more sensitive approach to measuring radiation injury will also facilitate studies beyond the direct scope of the present proposal. The reagents developed in the course of these studies, including recombinant single-chain antibodies that are specific for active repair complexes, should permit assessment of individual variation in the response to ionizing radiation. A rapid assay based on this technology will provide an inexpensive and practical means to determine variations in radiation sensitivity in populations of radiation workers and other individuals. This technology will also facilitate future, quantitative studies of differences between low-dose radiation and endogenous oxidative damage.

The ability to make direct measurements of the biological repair process triggered by low-dose radiation will contribute to informed decisions regarding the risks to workers and the public from exposures to low levels of radiation. This is one of the central concerns of the Low Dose Radiation Research Program.

Since double-strand break repair proteins are evolutionarily conserved, reagents developed in the course of this project may also prove useful for investigations in non-human species, including the free-living wildlife populations residing near sites contaminated with radionuclides. Long-term wildlife studies are in progress at the US DOE Savannah River Site in Barnwell County, South Carolina, which is located near our laboratory. In the future, it may be possible to adapt our technology to address questions of low-dose radiation effects in non-human species.

Technical Background

Figure 1. DSB Repair Pathway

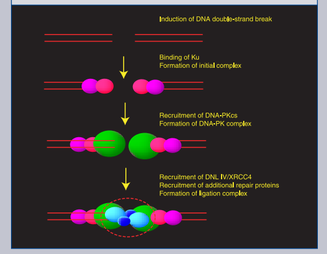
Knowledge of the biochemistry of double-strand break repair will facilitate the development of tools to allow the visualization of the active, assembled form of DSB repair complexes. Genetic and biochemical studies show that DSB repair involves a minimum of five polypeptides:

- Ku protein (70 and 80 kDa subunits)
- DNA-dependent protein kinase catalytic subunit (470 kDa)
- DNA ligase IV(100 kDa) and XRCC4 (40 kDa) tetramer complex

The diagram illustrates our current understanding of how these and other proteins assemble to form an active DSB repair complex. The first step is the induction of a DNA double-strand break by radiation or other means. The broken ends of the DNA are immediately bound by Ku protein to form an "initial complex." Ku protein recruits the DNA-dependent protein kinase catalytic subunit (DNA-PKcs) to form a catalytically active "DNA-PK complex."

The final steps are less well defined, but include the recruitment of a DNA ligase IV/XRCC4 complex. Other proteins are almost certainly involved; genetic analysis suggests that these may include proteins known as MRE11, RAD50, and NBS1, which help to trim away damaged DNA in preparation for rejoining. The DNL IV/XRCC4 complex, together with other proteins, forms the "ligation complex," which bridges the DNA ends and carries out the actual break rejoining.

Our strategy, as shown in the next panels, is to develop sensitive tools that allow the detection of **broken DNA ends** and **assembled repair complexes** *in situ* in irradiated cells.



Technical Approach: Current Work

Specific Aim 1:

The development of tools for *in situ* visualization of DSB repair complexes.

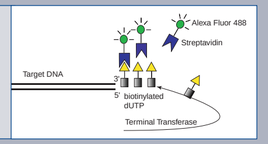
Direct visualization of DNA breaks.

Strategy

Figure 2. TdT Labeling Scheme

The diagram shows a strategy for *in situ* visualization of DNA double-strand breaks by a modified TdT dUTP nick-end labeling (TUNEL) method. Cells are irradiated, permeabilized, and incubated with biotinylated dUTP in the presence of terminal deoxynucleotidyl transferase. Cells are then incubated with a streptavidin-Alexa Fluor 488 conjugate.

The TdT is capable of labeling all types of 3' ends but has a preference for double-strand breaks with 3' protruding overhangs. We hope to develop various strategies to discriminate between single-stranded and double-stranded breaks.



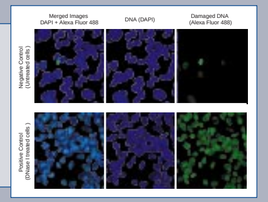
Proof of Concept

Figure 3. TdT Labeling of Cells Treated with DNase

The figure shows a control experiment demonstrating the ability to selectively label damaged DNA ends with TdT and biotinylated dUTP. Cells were permeabilized and labeled as shown in the preceding figure.

Top row shows negative control cells untreated with DNase. Bulk DNA is stained blue with DAPI; damaged DNA is stained green with Alexa Fluor 488; merged image shows staining with both agents. Note that there is little background of Alexa Fluor 488 staining except for one apoptotic cell within the field.

Bottom row shows cells subjected to limited treatment with DNase I after permeabilization but before staining. Note high level of green fluorescence, which reflects incorporation of biotinylated dUTP at sites of DNA damage.



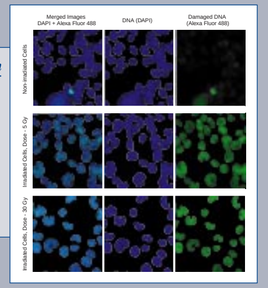
Preliminary Work

Figure 4. TdT Labeling of Cells Treated with Ionizing Radiation

The figure shows a preliminary experiment demonstrating the ability to detect DNA damage in irradiated cells with TdT and biotinylated dUTP. Cells were permeabilized and labeled as shown in the preceding figure.

Top row shows non-irradiated control cells. Bulk DNA is stained blue with DAPI; damaged DNA is stained green with Alexa Fluor 488; merged image shows staining with both agents. As in preceding figure, there is little background of Alexa Fluor 488 staining except for one apoptotic cell within the field. Second and third rows show cells subjected to 5 Gy and 30 Gy of ionizing radiation. Note high level of green fluorescence, which reflects incorporation of biotinylated dUTP at sites of DNA damage.

Initial experiments were performed at high doses to permit optimization of the methodology. Experiments at low doses are currently in progress.



Isolation of single-chain antibodies specific for assembled repair complexes.

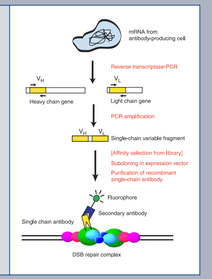
Strategy

Figure 5. Scheme for Cloning, Expression, and Use of Single-Chain Antibodies

The figure shows that messenger RNA is isolated from antibody-producing cells, which may be a monoclonal antibody-producing cell line or primary spleen cells from an immunized mouse. The RNA is subjected to reverse transcriptase-PCR amplification to separately produce DNA encoding antibody heavy and light chain variable regions. These are then linked in a series of PCR-mediated steps to produce a single-chain variable fragment.

A key step in the procedure is affinity selection of single-chain variable fragments that specifically recognize the active form of DSB repair complexes. For example, these may recognize protein-DNA interfaces, protein-protein interfaces, or novel conformational epitopes formed when the active repair complex is assembled.

Single-chain variable regions are then subcloned in an expression vector (containing additional antibody constant regions), expressed in bacteria, and used to stain irradiated cells by indirect immunofluorescence.

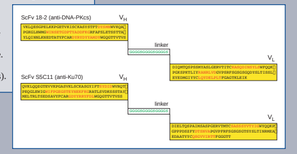


Proof of Concept

Figure 6. Cloning of Single-Chain Variable Fragment from Two Monoclonal Antibody Producing Lines

As a proof of concept experiment, we generated single-chain variable fragments from monoclonal antibodies 18-2 and S5C11, which are directed against DNA-PKcs and Ku protein, respectively. We are in the process of expressing these clones as single-chain antibodies in *E. coli*. These experiments provide the opportunity to identify and resolve technical issues before proceeding with the large-scale affinity selection.

Boxes denote heavy chain variable region, linker, and light chain variable region as indicated. Amino acid sequence is denoted as one-letter code. Red typeface indicates CDRs (complementarity determining regions).



Planned Future Activities

Specific Aim 2:

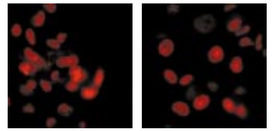
The use of the tools from Aim 1 to detect and characterize visible subnuclear structures associated with DSB repair.

- Experiments will be performed to correlate visible structures with biochemically-defined repair complexes.
- Strategies will be developed to prolong the lifetime of repair complexes to make them more easily visible.
- Immunoprecipitation experiments will be performed to identify additional proteins that contribute to formation of repair structures.

Example

Figure 7. Ionizing Radiation-Induced Foci

The figure shows an example of the type of visible subnuclear structures that previously have been shown to be induced by treatments that induce double-strand DNA breaks, the so-called ionizing radiation-induced foci. These foci are induced only at relatively high doses, and form 6-8 h after exposure, long after most breaks have been repaired. They may correspond to persistently unrepaired breaks. Our goal is to visualize similar types of structures that correspond to actual DSB repair complexes.



Specific Aim 3:

A demonstration of the practical utility of the tools and assays developed in Aims 1 and 2 by quantitative studies of DSB repair.

- Experiments will be performed to compare the responses at low and high radiation doses in different cell types.
- The ability of initial exposure to low-dose radiation to modify the response to a subsequent, higher dose will be determined.

Anticipated Benefits of the Project

- An understanding of the origin and fate of DNA double-strand breaks induced at low radiation doses.
- Tools for visualizing DNA breaks that can be used in future studies to assess individual variation in the response to radiation.
- Methods that are potentially applicable to the study of DNA double-strand break repair complexes in nonhuman species (including free-ranging wildlife).