VISUALIZATION OF DNA DOUBLE-STRAND BREAK REPAIR AT THE SINGLE-MOLECULE LEVEL

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INTRODUCTION/BACKGROUND

Exposure to low doses of ionizing radiation is universal. The signature injury from ionizing radiation exposure is induction of DNA double-strand breaks (DSBs). In mammalian cells, the first line of defense against DSBs is direct ligation of broken DNA ends via the nonhomologous end-joining pathway (reviewed in (1)). Because even a relatively high environmental exposure induces only a few DSBs per cell, our current understanding of the response to this exposure is limited by the ability to measure DSB repair events reliably *in situ* at a single-molecule level.

To develop a strategy to address this need, we considered three questions:

- What are the potential biomarkers that can be used to measure DSB repair?
- What changes do these biomarkers undergo upon exposure to ionizing radiation?
- What is the best technical approach to measure these changes to obtain the requisite sensitivity and specificity?

Fig. 1 shows the nonhomologous end-joining pathway. The heterodimeric Ku protein is the initial sensor of DNA breaks. Ku recruits the DNA-dependent protein kinase catalytic subunit (DNA-PKcs) to form an enzymatically active complex. The final step of phosphodiester bond formation is catalyzed by a mixed tetramer of DNA ligase IV and XRCC4. Other proteins, including some not yet identified, stabilize the synaptic complex, remove damaged nucleotides, and fill in remaining gaps. Exposure to ionizing radiation also causes phosphorylation of a variant form of histone H2A, known as H2AX, in the vicinity of the DSB (2). Any of the proteins in the DSB repair complex could be useful as a biomarker to detect individual repair events. Some proteins undergo focal redistribution to sites of DNA breaks. This can be detected by immunofluorescence microscopy. Other proteins undergo conformational changes. In principle, these could be detected by conformation-specific antibodies. Several proteins in the vicinity of the break undergo specific phosphorylation in response to radiation. These include H2AX, mentioned previously, XRCC4, and DNA-PKcs itself (3, 4).

Of the different physical and chemical changes that could be used to detect DSB repair responses, site-specific phosphorylation is easiest to detect and quantitate because it creates new, well-defined features (epitopes) on the surface of the chosen biomarker protein. This allows creation or selection of antibodies with high affinity for the phosphorylated form, and little or no affinity for the background population of unmodified proteins.

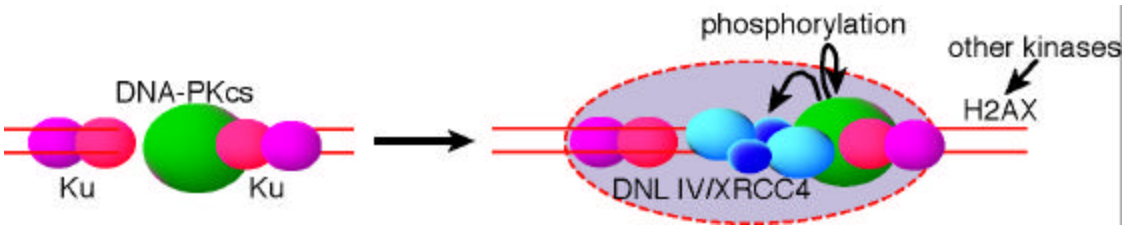

Figure 1. Nonhomologous end-joining pathway. Heterodimer Ku protein binds to a DSB, recruits DNA-PKcs. This complex serves as a platform for recruitment of additional proteins. DNA-PKcs phosphorylates itself, XRCC4, and perhaps other targets. Other radiation-inducible kinases phosphorylate histone H2AX.

MATERIALS/METHODS

ScFv selection. Biotinylated peptide with or without site-specific phosphorylation was used for selection of scFvs from a combinatorial murine phage display laboratory. Plaques representing individual phage particles were collected and used for production of soluble scFvs in the periplasmic compartment of *E. coli* strain HB2151 (Amersham Biosciences). ScFv was purified by anti-E tag affinity chromatography for use in ELISA and immunofluorescence assays.

Rabbit immunization. Peptide representing the C-terminal region of ϒ-H2AX was conjugated to keyhole limpet hemocyanin and used for immunization of rabbits. Animal work was performed by Biosynthesis, Inc. (Louisville, TX). Primary immunization was performed using 200 µg of conjugated peptide in Freund's complete adjuvant. Booster injections were performed with the same amount of peptide in Freund's incomplete adjuvant at days 14, 28, 42, and 56. Production bleeds were performed at days 42, 56, and 70. Immunoaffinity purification was performed serum collected at day 42 by passing the serum over a column of biotinylated ϒ-H2AX peptide bound to NeutrAvidin-agarose (Pierce, Rockford IL). Antibody was eluted from the column with Immunopure IgG elution buffer (Pierce).

ELISA and surface plasmon resonance assays. ELISA was performed using biotinylated H2AX peptides bound to a NeutrAvidin-coated multiwell plate (Pierce). Wells were incubated with scFv or rabbit antiserum as indicated in the legend to Fig. 3. Binding of scFv was detected using anti-E tag secondary antibody (Amersham Biosciences) and alkaline-phosphatase conjugated goat anti-mouse tertiary antibody. Binding of rabbit antibodies was detected using alkaline phosphatase-conjugated goat anti-rabbit secondary antibody. Wells were developed with Blue Phos substrate (KPL, Gaithersburg, MD). Surface plasmon resonance was performed using a Biacore X instrument (Biacore, Inc., Piscataway, NJ) where the ligand was of a biotinylated ϒ-H2AX peptide bound to a streptavidin-coated sensor chip, and the analyte was scFv at the concentrations indicated in Fig. 3. Data were analyzed assuming a 1:1 Langmuir binding model.

Immunofluorescence assays. SV40-transformed human fibroblasts (strain GM00637H, Coriell Cell Repository, Camden, NJ) were cultured on coverslips and exposed to ^{137}Cs irradiation (GammaCell 40 Exactor, Nordion, Ottawa, ON) at a rate of 1 Gy/min. Cells were allowed to recover for 30 min, fixed with formaldehyde, and stained with scFv or rabbit anti-ϒ-H2AX antibody as described in the Figure Legends. Staining with scFv was visualized using anti-E tag secondary antibody and AlexaFluor 488 or 594-conjugated tertiary antibody (Molecular Probes, Eugene, OR). Staining with rabbit antibody was visualized using AlexaFluor 594-conjugated goat anti-rabbit secondary antibody (Molecular Probes). Images were collected with a Zeiss DeltaVision microscope.

RESULTS/DISCUSSION

Our initial studies were performed with antibodies against the gamma isoform of histone H2AX, which has previously been shown to form discrete foci at sites of DNA double-strand breaks (2). One of the advantages of ϒ-H2AX is that multiple phosphorylation events occur in response to each DSB event. This natural biological amplification facilitates detection of the response to DSB events. The key technical requirement for detection of single-molecule events is optimization of signal-to-noise ratio.

We explored several technologies to determine which was the most effective. One of these used single chain antibodies selected against phosphorylated and nonphosphorylated C-terminal H2AX peptides. Figure 2A shows a comparison of the structure of single chain antibodies and naturally-occurring immunoglobulin G (IgG) molecules (5). In IgGs, the antigen-binding surface is part of a domain formed by the interface of heavy chain variable (V_H) and light chain variable (V_L) regions. In a single chain antibody, also referred to as a single chain variable fragment (scFv), the V_H and V_L sequences are joined by a flexible linker to form a single molecule.

ScFvs can be selected from a combinatorial library by phage display, without the need for immunization of an experimental animal. Their sequences can be modified to enhance stability and antigen-binding affinity, or to introduce fluorophores to facilitate detection. ScFvs can also be engineered for overexpression in microbial or mammalian host cells. Figure 2B shows the peptide used for selection of scFvs directed against ϒ-H2AX. Figure 2C shows a model of where the phosphorylated epitope, in the H2A C-terminal tail, resides with respect to the nucleosome core particle.

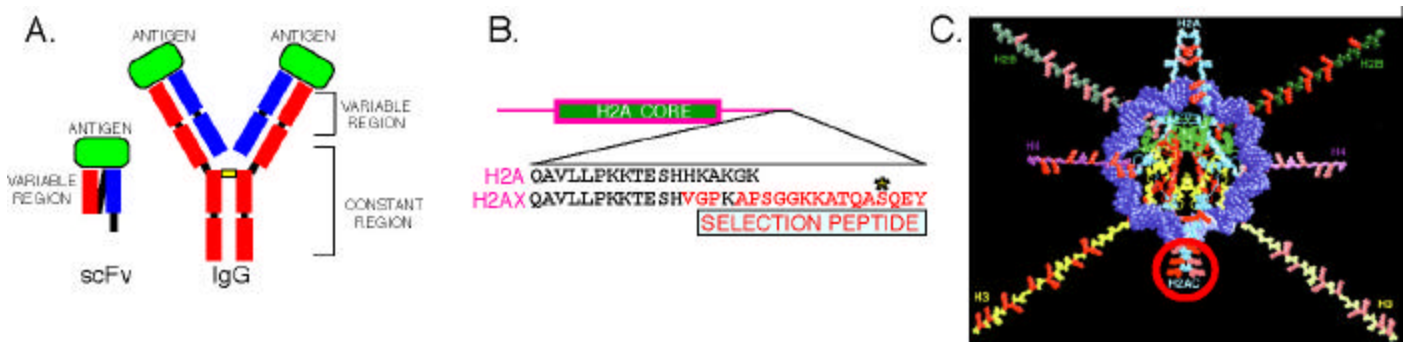

Figure 2. Strategy for production of scFvs against ϒ-H2AX. A. General structure of an scFv compared with a naturally occurring IgG. B. Peptide used for selection of scFvs from a combinatorial library. Asterisk denotes phosphoserine. C. Schematic showing location of phosphorylated region with respect to nucleosome core particles. One half of a core particle is shown. Flexible histone tails are modeled in fully extended conformation. Red circle denotes H2A tail. Figure reprinted from Nucleic Acids Research, Vol. 27, issue 3 (cover), copyright Oxford University Press. For details see article by Wolffe and Hayes (1999).

Ten scFvs were selected from a combinatorial phage display library using phosphorylated and nonphosphorylated peptides. They were tested in an enzyme-linked immunosorbent assay (ELISA), with results shown in Fig. 3. ScFvs selected with phosphorylated peptide were highly specific for phosphorylated peptide over nonphosphorylated peptide (D5, D10, D12, E6, E9, F1, H7). ScFvs selected with the nonphosphorylated peptide showed reciprocal specificity (C2, H11, 3G2). Binding of scFv F1 was further evaluated by surface plasmon analysis (Fig. 3C). The binding affinity was relatively weak (K_D approx 100 nM) and the dissociation was relatively rapid. We were not able to detect binding of this scFv to regions of double strand breaks *in situ* in irradiated cells, perhaps because of the weak affinity, or perhaps because the scFvs were unable to bind peptide in the native context (Fig. 3B). As a control, we performed staining under similar conditions with an scFv that binds with high affinity to DNA-PKcs. We saw robust staining, which was specific for DNA-PKcs-containing cells (Fig. 3D).

ScFvs selected from combinatorial libraries have not undergone the process of repeated mutation and selection, or "affinity maturation" that leads to production of high affinity antibodies *in vivo* in immunized animals. To obtain antibodies with potentially higher affinity, we immunized rabbits with carrier-conjugated ϒ-H2AX phosphopeptide. An ELISA showed that immune sera contained high-affinity antibodies capable of reacting with H2AX peptide, but there was little or no specificity for the phosphorylated form (Fig. 3). There was also only modest specificity for staining nuclei of irradiated versus nonirradiated cells (data not shown). To enrich for the phosphorylation-specific component, we performed affinity chromatography using a ϒ-H2AX phosphopeptide column. This produced a highly specific, high-affinity antibody preparation that could be used for selective immunostaining (Fig 4).

Although induction of H2AX phosphorylation has been well characterized at higher radiation doses, its use as a biomarker for low dose exposure has not been systematically explored. Fig. 4 presents results of a preliminary experiment in which human cells were exposed to a dose of 10-100 cGy. Using anti-ϒ-H2AX

antibodies, we observed a dose-dependent increase in foci over the range 10-100 cGy. Quantitation shows that the number of foci corresponds well with the number of breaks anticipated based on extrapolation from higher doses (roughly 3-30 breaks/ cell). This suggests that each focus represents the response to an individual DSB repair event, even at very low doses that introduce only a few breaks per cell. In the future, we plan to perform similar experiments using other radiation-specific markers. Coincident staining with several markers is expected to reduce sporadic background and will increase confidence that each focus represents a response to a single-molecule breakage event.

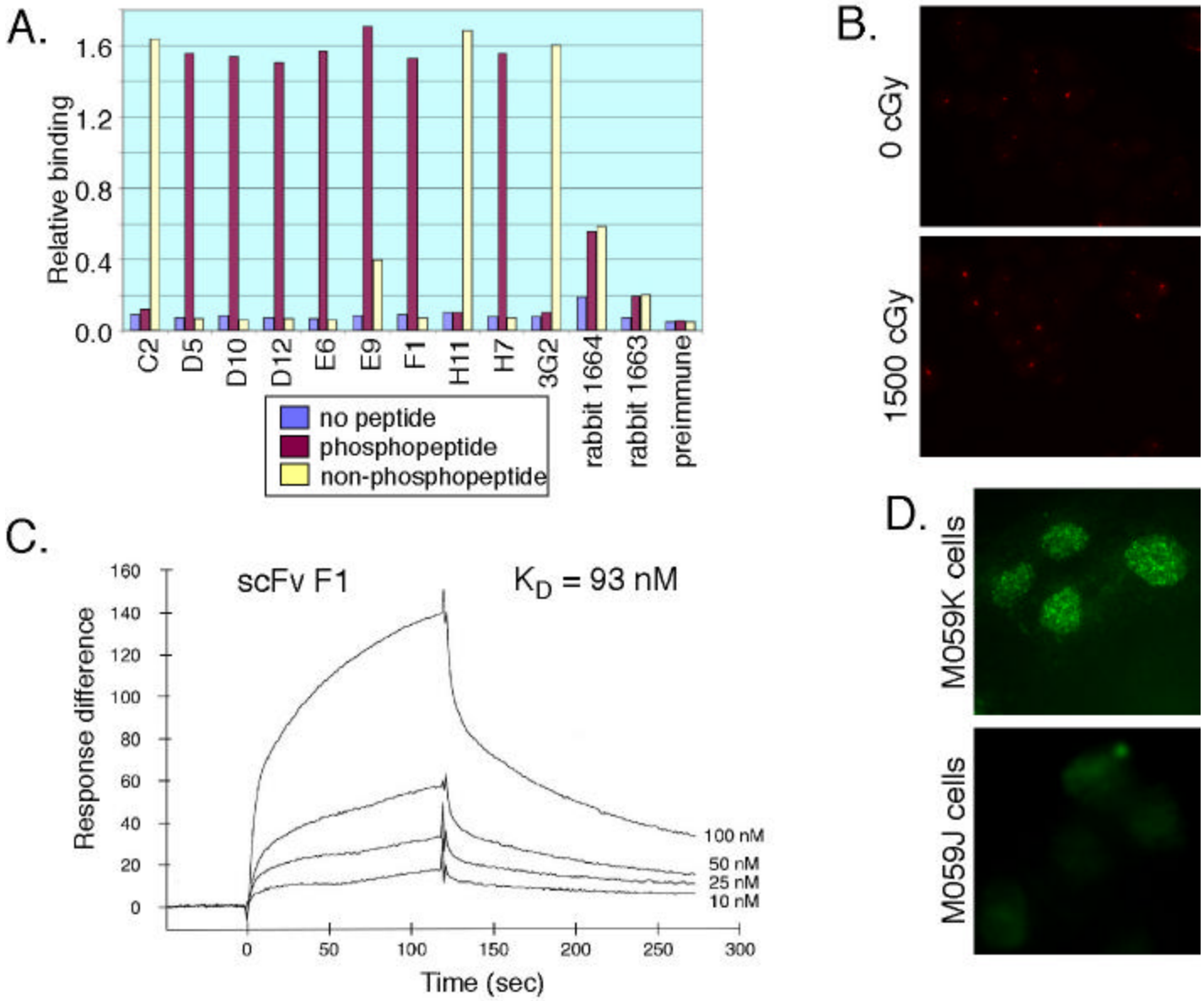

Figure 3. Characterization of scFvs selected against H2AX peptides. A. ELISA assays performed as described in Materials/Methods. B. Immunofluorescence assays to test binding of scFv to nonirradiated and irradiated HeLa cells. Faint red dots do not increase in number upon irradiation are believed to be nonspecific. C. Measurement of kinetic parameters for binding of scFv F1 to phosphorylated peptide. D. Control experiment demonstrating ability to detect binding of scFv 18-2, directed against DNA-PKcs, under similar staining conditions. Human glioma cell line M059K contains DNA-PKcs, and M059J is a DNA-PKcs-negative variant.

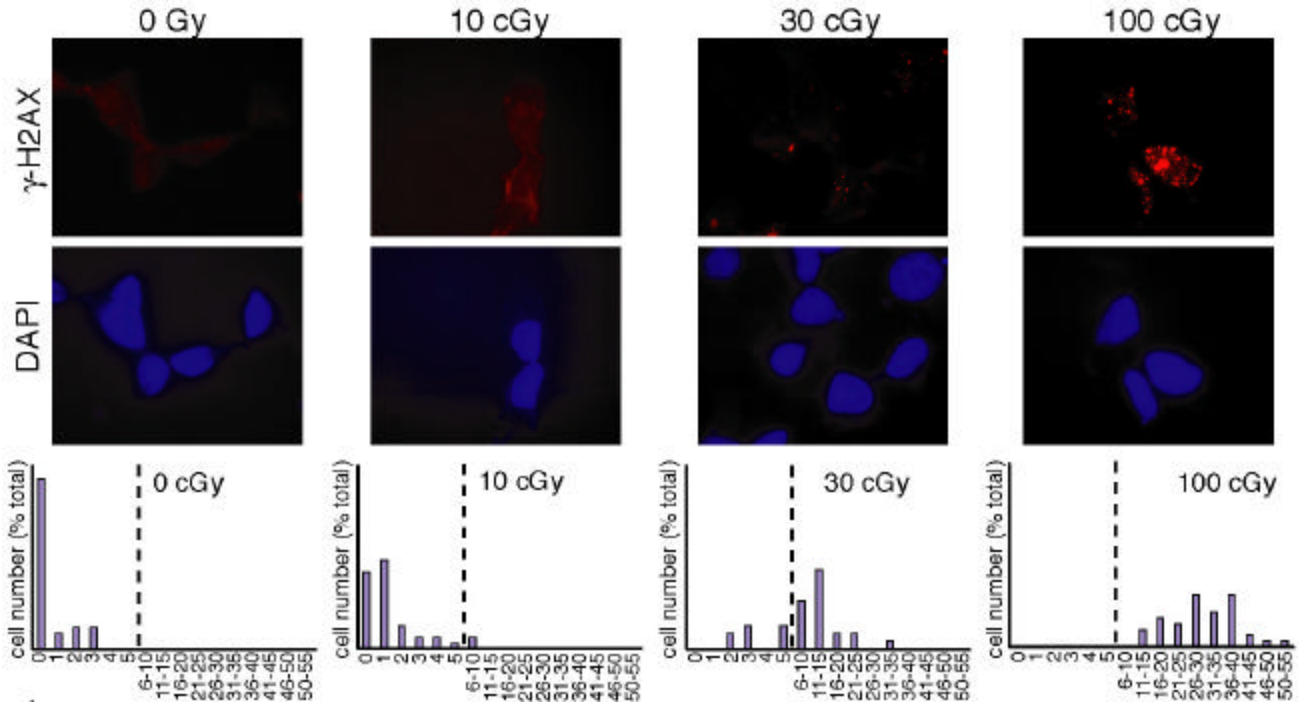

Figure 4. Detection and quantitation of γ-H2AX foci at low radiation doses. Irradiation and immunostaining of human fibroblasts was performed as described in Materials/Methods. DAPI counterstaining indicates position of cell nuclei. Approximately 30 cells were scored at each dose.

SIGNIFICANCE

The availability of methods to measure the response to small numbers of DSB events induced in single cells by environmentally relevant doses of radiation is potentially useful for a variety of purposes:

- Investigation of the mechanism of the response to low radiation doses that induce only one or a few breaks per cell. For example, although DNA-PKcs activity is required for survival after high dose irradiation, there is no proof yet that it is required for repair of small numbers of breaks induced at low doses. Investigation of this question by microinjection of activity-neutralizing antibodies into living cells is in progress.
- Biologically-based dosimetry. Existing dosimetry methods measure physical deposition of energy in tissue. A biologically-based method can be used to measure the actual number of breaks induced in cells of target organs, the time course of repair, and the persistence of unrepaired breaks.
- Extension of mechanistic and dosimetry studies to whole animals, including laboratory species or free-ranging wildlife. γ-H2AX and the protein components of the nonhomologous end-joining pathway are highly conserved between species. Experiments are planned to test our approach in a fish model.

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