Cell cycle dependent phosphorylation and focus-formation of histone H2AX in X-irradiated malignant B-lymphocyte cell lines

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2. Abbreviations

- APAF1 Apoptotic protease activator factor 1
- AT Ataxia telangiectasia
- ATM Ataxia telangiectasia mutated protein
- ATR ATM and RAD3-related protein
- BAX BCL2 associated X protein
- BCL2 B-cell CLL/Lymphoma 2
- BID BH3 interacting domain death protein
- BRCA1 Breast cancer susceptibility protein 1
- BRCA2 Breast cancer susceptibility protein 2
- CCN Cyclin
- CDC25A Cell division cycle 25A protein
- CDK2 Cyclin-dependent kinase 2
- CDKN1A Cyclin-dependent kinase inhibitor 1A
- CHK2 Checkpoint kinase 2
- DNA-PK DNA dependent protein kinase
- DNA-PKcs Catalytic subunit of DNA-PK
- DSB DNA double-strand break
- HR Homologous recombination
- IR Ionizing radiation
- MDC1 Mediator of DNA damage checkpoint protein 1
- MRE11 meiotic recombination 11 homolog protein
- MRN MRE11/RAD50/NBS1
- NBS1 Nijmegen breakage syndrome 1 protein
- NHEJ Non-homologous end joining
- PIKKs Phosphatidylinositol 3-kinase like kinases
- RPA Replication protein A
- SSB DNA single-strand break
- 53BP1 TP53 binding protein 1
3. Introduction

The genetic information for the identity and function of eukaryotic cells resides within their DNA. Thus, maintenance of genomic stability is of crucial importance for all life. Because the environment delivers a continual onslaught of insult to our genetic material, a series of defensive mechanisms are required if humans or other species are to survive. Any damage to cellular DNA is responded to instantly by an assortment of biochemical pathways whose functions are to detect DNA damage and effect its repair.

Ionizing radiation (IR), as an effective physical agent for cancer therapy, targets primarily DNA and produces an array of lesions that include single-strand breaks (SSBs), base alternations, and double-strand breaks (DSBs). Among these types of IR-induced lesions, emphasis is currently placed on DNA DSBs as the most dangerous, as chromosomal breakage may result in loss of genetic integrity.\(^1,2,3\) This is particularly important when associated with abnormal regulation of the cell cycle and may give rise to cancer or hereditary diseases.\(^4,5\) Cancer is very often associated with the accumulation of genomic aberrations including large deletions, inversions or translocations, which may be consequences of DSBs.\(^6\) In response to this threat eukaryotic cells have evolved specialized and redundant molecular mechanisms to rapidly and efficiently sense DSBs, signal their presence, and bring about their repair. Higher eukaryotic cells primarily repair DSBs by one of two distinct separable pathways, namely non-homologous end joining (NHEJ) and homologous recombination (HR).\(^7,8\)

However, abundant complex DSBs will present a severe challenge to the repair machineries with repeated attempts at repair likely to result in genomic instability. For multicellular eukaryotes at least, struggling to complete repair is problematic, whereas removal of severely damaged cells is a more sensible strategy. Normally, cells respond to such irreparable or extensive DSBs by undergoing programmed cell death, termed apoptosis. Apoptosis is an ordered disassembling and recycling of the entire cell in such a way as to have minimal impact on surrounding cells.\(^9\) Some cells, especially cancer cells, frequently acquire defects in the molecular regulatory mechanisms of response to IR-induced
Thus, an understanding of IR-induced DNA DSB signaling and repair pathway is not only of considerable academic interest but may also have a major impact on our understanding of various disease states, and may suggest how these can be treated or diagnosed more efficiently.

3.1 Ionizing radiation (IR) induced DNA double strand breaks (DSBs)

Ionizing radiation (IR) has been used to treat human malignancies since the early part of the 20th century. IR is electromagnetic radiation, such as x-rays and gamma rays, which is capable of producing ions in its passage through matter. The energy dissipated per ionizing event is more than enough to break a strong chemical bond. The absorbed dose of IR is measured as the gray (Gy, 1 joule of energy absorbed by 1 kilogram of material). The effects of IR on DNA have been studied for many years. Such studies are justified by the central role DNA plays as the major cellular target.

DNA is a large molecule with a well-known double helix structure. It consists of two strands, held together by hydrogen bonds between the bases. The phosphodiester backbone of each strand consists of alternating sugar and phosphate groups. Attached to this backbone are four bases; thymine, cytosine, adenine, and guanine, the sequence of which the genetic code. The bases in opposite strand must be complementary; adenine pairs with thymine, and guanine pairs with cytosine. Thus, if cells are irradiated with IR, result in disruption of the phosphodiester backbone on both strands of the DNA double helix.

Among the many lesions produced by IR, it is generally accepted that the DNA DSBs are the most important for radiobiological effects such as chromosome aberrations, cell death, and carcinogenesis. DNA DSBs are generated when the two complementary strands of the DNA double helix are broken simultaneously at sites that are sufficiently close to each other that base pairing and chromatin
structure are insufficient to keep the two DNA ends juxtaposed (fig. 1). It is estimated that each gray unit (1Gy) of radiation produces roughly 20 - 40 DSBs per diploid genome. As a consequence, the two DNA ends generated by a DSB are liable to become physically dissociated from another, making ensuing repair difficult to perform and providing the opportunity to inappropriate recombination with other sites in the genome. Therefore, eukaryotic cells have evolved highly conserved systems to rapidly and efficiently detect IR-induced DSBs, signal their presence and then bring about their repair.

Figure 1. Schematic presentation of IR-induced DNA DSB. The figure was obtained from Hall.
3.2 The sensing of and response to IR-induced DNA DSBs

Cells respond to IR-induced DNA DSBs through the actions of systems that detect the DNA lesion and then trigger various downstream events. These pathways can be viewed as classical signal-transduction cascades in which a signal (DSB) is detected by a sensor (DNA-damage binding protein) that triggers the activation of a transducer system (e.g. a protein kinase cascade), which amplifies the signal by targeting a series of downstream effectors of the DNA-damage response (fig. 2). One well characterized response to DNA DSBs is for cells to slow down progression through the cell cycle, which is thought to provide time for repair, thus preventing mutations from being propagated. These cell-cycle checkpoints are believed to prevent the replication of damaged DNA (G1/S and intra-S checkpoint) or segregation of damaged chromosome (the G2/M checkpoint). Clearly, such systems need to be exquisitely sensitive and selective, as they must be triggered rapidly and efficiently by low numbers of, and maybe just one, chromosomal DNA DSB.

Figure 2. Schematic representation of cellular response to DNA DSB, share the characteristics of a signal transduction pathway.

Sensor proteins recognize DNA damages and function to signal the presence of these abnormalities and initiate the biochemical cascade (A). Transducers are typically protein kinases that relay and amplify the damage signal from sensors by phosphorylating other kinases or downstream target proteins (B). Effector proteins include the ultimate downstream targets of the transducer protein kinases. Modification of effector proteins will then mediate the inhibition of cell cycle progression (C). The figure is modified from Belli et al. 

DNA

DBS

Sensors

Transducers

Effectors

Cell cycle checkpoints DNA repair Apoptosis
3.2.1 Sensing DNA DSBs

The first critical step in the cellular response to DSBs is to sense these lesions, by proteins that interact directly with the damaged DNA. In addition, it is suggested that the interaction between the sensor proteins and the damaged DNA site may be facilitated by a conformational change that causes an increased binding of proteins to DNA. Recent structural studies have described the major cellular sensor proteins of IR-induced DSBs; the MRN protein complex (composed of MRE11/RAD50/NBS1 proteins in humans), the KU proteins (KU70 and KU80), and the phosphatidylinositol 3-kinase like kinase (PIKK) family members; which include the ataxia-telangiectasia mutated (ATM) and the catalytic sub-unit of the DNA-dependent protein kinase (DNA-PK<sub>CS</sub>).

The importance of sensing DSBs is clear from the profound phenotypes associated with mutations in genes encoding proteins that participate in the cellular DSB response. Perhaps one of the most important recent developments in the field has been insight into the mechanism of ATM activation in response to IR-induced DNA DSBs. ATM is encoded by a gene that is mutated in individuals with ataxia telangiectasia (AT), which is characterized by progressive neurological degeneration, growth retardation, specific immunodeficiency, a high sensitivity to IR, and an increased incidence of malignancy. One model for ATM activation by phosphorylation, proposes that ATM itself is the direct sensor of damaged DNA, and that it does not require upstream activators. However, several recent reports have placed the MRN complex as a DSB sensor and an upstream activator of ATM. Activation of ATM is defective in cells that are compromised for nijmegen brekage syndrome1 (NBS1) or mitotic recombination 11 homolog (MRE11) protein, and phosphorylation of some downstream targets of ATM is partially dependent on a functional MRN complex. Furthermore, the nuclease activity of the MRE11 protein is required for activation of ATM, suggesting that DNA DSBs may require processing prior to activation of ATM. Also, ATM-mediated phosphorylation of MRN on NBS1 occurs at sites of DNA damage. Together, these studies suggest an attractive model in which (i) the MRN complex binds directly to damaged DNA, (ii) ATM is recruited and activated through phosphorylation and monomerization, and (iv) kinase-active,
monomeric ATM enable phosphorylation of more distal substrates, involved in the cell-cycle checkpoints (fig. 3).36

Analogous to the relationship between MRN and ATM, the KU proteins (KU70 and KU80) are required for activation of DNA-PKCS upon IR-induced DSBs, but predominantly those induced in G1 phase cells.45,46 In addition, MRN/ATM and KU/DNA-PKCS may serve critical, overlapping roles in DSBs detection, particularly during the G1 phase of the cell cycle.23 In contrast to MRN/ATM, KU and DNA-PKCS do not appear to activate proteins involved in the cell cycle checkpoint pathway.29 DNA-PKCS can bind weakly to DNA ends, but this activity is greatly stimulated and stabilized by a heterodimer formed by KU70 and KU80 subunits.29,47 Recent structural studies have provided insight into how KU proteins bind to sites of DNA damage. In the structure reported by Walker et al. 48, the KU heterodimer forms a ring through which the DNA is threaded. One side of this ring forms a cradle that protects one face of the DNA double helix (fig. 3). The structure helps to explain many of the biochemical data regarding KU activity, including the observation that KU makes contact almost exclusively with the sugar-phosphate backbone of the DNA and that the high-affinity binding of KU to DNA ends is sequence independent.47 Taken together, these results suggest a model in which the two KU heterodimers, KU70 and KU80, bind too and bridge the broken DNA ends of IR-induced DSBs.49,50 DNA-PKCS is then recruited to the break where it may stabilize the bridge, producing the DNA dependent protein kinase complex (DNA-PK), and thus recruit and activate factors needed for DNA DSBs repair pathway by NHEJ.48,51
3.2.2 The G₁/S checkpoint

To prevent entry into S phase with DNA DSBs, cells traversing G₁ phase activate the checkpoint kinase 2 (CHK2) by ATM⁵² which, in turn, target two critical effectors operating in distinct branches of the G₁ checkpoint, the cell division cycle 25A protein (CDC25A) phosphatase and the TP53 transcription factor (fig. 4).⁵²,⁵³ Importantly, despite the phosphorylation of CDC25A and TP53 by checkpoint kinases occurs rapidly and simultaneously, the impact of these events on cell cycle machinery is fast in case of the CDC25A cascade which, unlike the slower-operating TP53 pathway, does not require transcription and accumulation of newly synthesized proteins.⁵⁴
The phosphorylation of CDC25A will result in its enhanced ubiquitination and proteasome-mediated degradation, thereby preventing the CDC25A-mediated dephosphorylation of cyclin-dependent kinase 2 (CDK2), the catalytic subunit of cycline E (CCNE)/CDK2 and CCNA/CDK2. Such inhibition of CDK2 activity blocks the assembly of pre-replication complex, which result in preventing initiation of DNA synthesis. The checkpoint pathway that targets CDC25A is implemented rapidly, it operates independently of the TP53 status, and it is relatively transient, capable of delaying cell cycle progression for only several hours.

On the other hand, the complementary mechanism responsible for the prolonged maintenance of the G₁ cell-cycle arrest in response to extensive DNA DSBs reflects the other branch of the G₁ checkpoint, dependent on TP53. In contrast to CDC25A, TP53 is phosphorylated not only by CHK2 but also directly by the ATM protein. In addition, the ubiquitin ligase MDM2 that normally binds TP53 and ensures rapid TP53 turnover, is also targeted after DNA DSBs by ATM. Collectively, these DNA-DSBs-induced modifications of TP53 itself and its negative regulator MDM2 contribute to stabilization and accumulation of the TP53 protein, as well as to its increased activity as a transcription factor. The key effector of TP53-dependent transcription in relation to G₁ arrest is the CDKN1A, an inhibitor of cyclin dependent kinases. Accumulation of CDKN1A after DNA damage to threshold levels capable of blocking the G₁ to S –promoting CCNE/CDK2 complex leads to a sustained cell cycle arrest (fig. 4).

3.2.3 The intra-S checkpoint

The intra-S checkpoint activated by DSBs causes only a transient, reversible delay in cell cycle progression, mainly by inhibition the initiation of DNA replication. Thus unlike the G₁/S checkpoint, the intra-S phase response to DNA damage lacks the sustained maintenance phase of the cell-cycle arrest, and it is independent of TP53.

The MRN complex is required for normal function of the intra-S checkpoint. The early recruitment of NBS1 as part of the MRN complex to sites of
DNA damage (DSBs) and the processing of DNA breaks by this complex seem independent of ATM\(^{43,62,63}\), while the phosphorylation of NBS1 by ATM may switch on the function of NBS1 as a checkpoint transducer. Another substrate of ATM that appears associated with the checkpoint role of NBS1 is the SMC1 (structural maintenance of chromosome 1) protein complex (fig. 4), which is a component of the cohesin complex that is required for the cohesion of sister chromatids\(^{62}\). Interference with phosphorylation of SMC1 impairs the intra-S phase checkpoint, yet the downstream events regulated by NBS1 and/or SMC1 that may inhibit DNA synthesis remain elusive.\(^{62}\)

### 3.2.4 The G\(_2\)/M checkpoint

The G\(_2\)/M checkpoint prevents cells from initiating mitosis when they experience DNA DSBs during G\(_2\), or if they progress into G\(_2\) with some unrepaired damage inflicted in previous S phase or even G\(_1\) phase. However, the central event in activation of the G\(_2\)/M checkpoint is inhibition of the mitosis-promoting phosphatase CDC25C.\(^{64}\) Studies of cells deficient in ATM or CHK2 kinase have shown that the ATM-CHK2 pathway is responsible for activation of the G\(_2\)/M checkpoint in response of IR-induced DNA DSBs.\(^{65}\) ATM activates CHK2 by phosphorylation, and activated CHK2 thus phosphorylates CDC25C\(^{52}\) and thereby creates a binding site for 14-3-3δ effector protein.\(^{66}\) The interaction with 14-3-3δ prevents CDC25C from dephosphorylating and activating the mitotic kinase complex CCNB/CDK1, thus effectively blocking cells with DSBs damage from entering mitosis (fig. 4).\(^{38,67}\)
Figure 4. overview of the response to IR-induced DNA DSB.
(See main text for details). The figure is modified from Sancar et al.\textsuperscript{21} and Iliakis et al.\textsuperscript{65} Apoptosis and DNA repair pathways will be discussed in next sections.

3.3 The connection between DNA DSB signaling and repair: phosphorylation of histone H2AX

In mammalian cells, DNA is densely packaged within chromatin, of which the basic unit is the nucleosome. Nucleosomes consist of approximately 200 base pair of DNA wrapped around two molecules each of the H2A, H2B, H3, and H4 core histones. Nucleosomes are tethered together by the H1 linker histone and assembled into higher order chromatin structures.\textsuperscript{13,68} Thus, an essential component of the cellular response to DSBs must be the modulation of higher order chromatin structure to allow NHEJ and HR repair proteins access to the
liberated DNA ends. The mechanism through which these changes in chromatin structure are promoted remain to be elucidated, however, an emerging principal appear to involve the covalent modifications of core histones.\textsuperscript{69,70,71}

The only chromatin modification in response to IR-induced DNA DSBs that has been extensively characterized in mammalian cells is phosphorylation of the histone H2A variant, H2AX.\textsuperscript{72,73} This phosphorylation of histone H2AX, yielding a specific modified form named $\gamma$-H2AX, can be detected within minutes (1min-10min) after the introduction of DSBs.\textsuperscript{74} In humans H2AX represents 2%-25% of the total H2A pool\textsuperscript{75,76}, and is incorporated apparently randomly into nucleosomes.\textsuperscript{76} H2AX serves critical functions in the cellular response to DSBs\textsuperscript{75,77}, as H2AX-deficient cells exhibit increased IR-sensitivity and elevated levels of spontaneous genomic instability.\textsuperscript{78,79,80}

Upon IR-induced DSBs, ATM and DNA-PK rapidly phosphorylates serine 139 residue present within the conserved carboxy terminal SQE motif of H2AX, to form $\gamma$-H2AX foci along flanking two megabase chromatin regions.\textsuperscript{74,75,76,81,82,83} In addition, it has been reported that H2AX phosphorylation also occurs in S and G\textsubscript{2} nonirradiated cells.\textsuperscript{16} This phosphorylation is mediated by ATR (ATM and RAD3-related protein), which is also related to the PIKKs.\textsuperscript{82,84} Recent studies had observed a close correlation between the number of $\gamma$-H2AX foci and the number of expected DSBs after IR-treatment.\textsuperscript{70,75} By using a fluorescent antibody specific for the phosphorylated form of $\gamma$-H2AX confirmed that the number of these foci are comparable to the number of DNA DSBs.\textsuperscript{14,85,86} Subsequent to H2AX phosphorylation, recent studies have reported a rapid assembly of DSB response proteins that occurs within the context of $\gamma$-H2AX foci.\textsuperscript{70,87} These results suggested that $\gamma$-H2AX is thus required for efficient accumulation of several checkpoint and DNA repair proteins to the break site, including; the mediator of DNA damage checkpoint 1 (MDC1)\textsuperscript{70}, TP53 binding protein 1 (53BP1)\textsuperscript{70}, breast cancer susceptibility protein 1 (BRCA1)\textsuperscript{70,78}, the MRN complex proteins\textsuperscript{89,90}, and RAD51 (fig. 5).\textsuperscript{78,70,91} The recruitment of these proteins to $\gamma$-H2AX has been believed to facilitate DNA DSB repair by increasing the local concentration of checkpoint and repair proteins into complexes near the lesion.\textsuperscript{70,92} Thus, the formation and rate of loss of $\gamma$-H2AX foci has recently provided an extraordinarily
sensitive technique to monitor DSB formation and repair.\textsuperscript{70,85,93,94} However, a variety of recent lines of evidence have suggested a second novel, specific 'anchoring' function for H2AX in suppressing aberrant processing of DSBs by keeping broken DNA ends tethered together.\textsuperscript{77,80,95} In the context of this model, following IR treatment, multiple DSBs along the same chromosome or on different chromosomes may occur simultaneously within a single cell. Thus, to avoid intra-chromosomal rearrangements (deletions) and inter-chromosomal rearrangements (translocations) and, thereby, maintain genomic stability, eukaryotic cells appear to have evolved mechanisms to prevent the ligation of liberated DNA ends between different DSBs. For example, high dose IR-induced DSBs frequently leads to translocations in NHEJ-deficient cells, indicating that NHEJ favors re-ligation of ends directly across DSBs and is suppressed from occurring between different DSBs.\textsuperscript{96} This finding suggested that there are factors, potentially in chromatin, that hold broken ends together until they are properly joined.\textsuperscript{77,96} Thus, by the chromatin associated changes that could promote proper re-ligation of broken ends to prevent translocation, γ-H2AX has been proposed to serve as an anchor for the formation of multiple DNA-protein-protein-DNA complexes involving MDC1, 53BP1 and the MRN complexes that would promote chromatin compaction and help prevent broken chromosomal DNA ends from separating before they are joined.\textsuperscript{77} This specific 'anchoring' at general DSBs could prevent irreversible dissociation and subsequent mis-repair of DSBs between chromosomes, thereby preventing generation of translocations. This function of γ-H2AX may, at least in part, underlie its contribution to suppression of translocations and further development of tumors.\textsuperscript{77,97}
Figure 5. Model of the DSB response cycle mediated by γ-H2AX;

Undamaged chromosome and inactive ATM dimer (A). IR-induced DNA DSB lead to modification of chromatin, activation (B), and recruitment of both ATM and MRE11/RAD50/NBS1 (MRN) (C). Both H2AX phosphorylation and thus more chromatin modification (D) is followed by recruitment of cell cycle checkpoint and repair proteins (e.g. BRCA1, 53BP1, MDC1) to the growing focus, and their ATM dependent phosphorylation (E). Disassembly of the focus proteins after DSB repair, and ATM inactivation (F). The figure was obtained from Van den et al.\textsuperscript{25}
3.4 Repair of IR-induced DNA DSBs

Potentially lethal DSBs are repaired by NHEJ mainly in G₁/early S cell-cycle phase and by HR, in late S/G₂ cell-cycle phase.⁹⁸,⁹⁹ The fundamental difference in these pathways is the requirement for a homologous DNA sequence; HR repairs DSBs by retrieving genetic information from an undamaged homologue (sister-chromatid), and this requires DNA sequence homology. In contrast to HR, NHEJ rejoins DSBs via direct ligation of the DNA ends without any requirement for sequence homology. As a consequence of the mechanisms, the accuracy of repair constitutes a crucial difference between the two pathways; while HR ensures essential accurate repair of DSBs, NHEJ rejoin broken DNA in a manner that is almost always mutagenic.⁷,⁸,⁹⁶

3.4.1 Repair by non-homologous end joining pathway

For a long time NHEJ has been regarded as the major mechanism for DSB repair in mammalian cells.¹⁰⁰ Genetic studies, using radiosensitive mammalian cell lines defective in DSBs rejoining and with mutations in genes that encode components of NHEJ, have been useful in identifying several proteins involved in this DSB repair pathway.¹⁰¹,¹⁰²,¹⁰³,¹⁰⁴

Although the molecular mechanism of the NHEJ pathway is still not resolved in detail, it is to be expected that the direct joining of two DNA termini requires at least four steps, namely (i) detection of a DSB (ii) formation of a molecular bridge that holds the DNA ends together, (iii) a processing procedure that modifies non-matching and/or damaged DNA ends into compatible and ligatable ends, and (iv) the final ligation (fig. 6).⁷,¹⁰⁵ As mentioned in section 3.2.1, in the initial step, the KU70/KU80 heterodimer recognize and binds to the DSB and has, at least, three major functions: keeping DNA ends in proximity⁴⁵,¹⁰⁶, protecting DNA ends from extended degradation¹⁰⁷, and recruiting the other components of the NHEJ mechanism, such as DNA-PKCS.⁴⁷

DNA-PKCS interacts with the KU complex bound to DNA ends, a process that leads to activation of DNA-PKCS kinase activity (fig. 6), resulting in the
formation of the DNA-PK complex.\textsuperscript{47,108,109} Two molecules of DNA-PK\textsubscript{CS}, one at each side of the DSB may hold the ends of DNA together prior to ligation.\textsuperscript{110}

Thus, processing of DNA ends must occur prior to ligation since IR-induced DSBs frequently contains 3’ and 5’ overhangs rather than blunt ends, as well as 3’-phosphate groups that must be processed or removed prior to ligation. In addition, IR induces damage to bases and/or ribose units in the vicinity of the break, so-called, clustered DNA damage\textsuperscript{3} that need to be repaired. Processing of the DNA ends may involve the MRN complex, participating via its exonuclease activity.\textsuperscript{111,112} Finally, the DNA ends ligation is subsequently carried out by the XRCC4/DNA ligase IV complex.\textsuperscript{113} However, during the end-joining process, limited degradation of the DNA ends can lead to deletion or insertion of nucleotides or DNA fragments; NHEJ is thus, a potentially error-prone process (fig. 6).\textsuperscript{7,114,115}

### 3.4.2 Repair by homologous recombination pathway

In contrast to NHEJ, HR promotes accurate DSB repair by using an extended, undamaged homologous sequence as template.\textsuperscript{8} HR is thought to be particularly important for DSB repair in S and G\textsubscript{2} cells where a sister chromatid would be available to provide a template.\textsuperscript{99,116,117} The mechanism of HR repair pathway is only partially understood, though many models have been proposed. There are a large number of proteins involved in the recognition of and response to DSBs in the context of HR.\textsuperscript{8} The molecular basis and genetic requirements of HR were initially defined by studies in bacteria and yeast\textsuperscript{118,119,120}, but it has become clear that this pathway is well conserved in higher organisms, such as mammals. In brief, genetic analysis of mammalian homologous have been described which include a set of eleven genes; \textit{RAD50, RAD51, RAD52, RAD54, XRCC2, XRCC3, RAD51B, RAD51C, RAD51D, MRE11} and \textit{NBS1}, collectively referred to as the “RAD52 epistasis group”.\textsuperscript{117,120,121,122}

The events of HR are complex and, based on analyses of HR under various biological circumstances and in different organisms.\textsuperscript{117,120} An outline of one model is given in figure 5. The first step of HR pathway is believed to be the recognition
of the DNA DSB ends by ATM\textsuperscript{123,124} and the MRN complex\textsuperscript{111} (see section 3.2.1), respectively.\textsuperscript{111,121} ATM has been reported to mediate the accumulation of HR proteins to the break site by γ-H2AX (see section 3.3.).\textsuperscript{70,123} However, evidence suggests that DSB end processing by the MRN complex is also a major event for initial binding of the RAD52 epistasis group members to the break site.\textsuperscript{111} The MRN complex with a 5´-3´ exonuclease activity that digest several nucleotides from both strands, producing 3´ single stranded DNA (ssDNA) overhangs, preparing DNA for HR (fig. 6).\textsuperscript{111}

The ensuing 3´ single-stranded DNA tails are then bound by RAD51 in a process that is influenced by a range of other proteins, including replication protein A (RPA), RAD52 and RAD54 proteins.\textsuperscript{121,125} RAD51 together with other RAD52 epistasis group members accumulates on ssDNA until the genomic search for homologous sequences is successful and the homologous donor DNA molecule is invaded.\textsuperscript{117} After strand invasion, the 3´ terminus of the damaged DNA molecule is then extended by a DNA polymerase β\textsuperscript{126} that copies information from the undamaged partner, and the ends are ligated by DNA ligase IV.\textsuperscript{127} Finally, the DNA crossovers are resolved by cleavage and ligation to yield two intact DNA molecules (fig.6).\textsuperscript{8}

However, it has also been established strong links between HR and the breast cancer susceptibility proteins, BRCA1 and BRCA2, which do not appear to have direct homologues in yeast.\textsuperscript{128,129,130} Specifically, loss of function of either BRCA1 or BRCA2 in mammalian cells markedly reduces the efficiency of accurate homology directed DNA repair.\textsuperscript{131,132,133,134} Furthermore, mutations of BRCA2 stimulates error-prone homology directed repair of DNA DSBs that have been generated between repeated sequences.\textsuperscript{135} It is not yet clear exactly how these effects are brought about but they may reflect the binding of BRCA1 and BRCA2 to RAD51.\textsuperscript{136} It has been reported that BRCA1 and BRCA2 directly interacts to and modulates the nuclear localization and DNA binding properties of RAD51\textsuperscript{137,138}, probably allowing RAD51 to form foci at sites of DNA damage within the cell.\textsuperscript{130,139,140}
Figure 6. Schematic representation of IR-induced DNA DSB (A) repair by NHEJ (B) and HR (C) in mammalian cells.

In NHEJ, KU70/80 bind to the DNA ends (1). This is followed by the recruitment of DNA-PKcs, forming DNA-PK complex, which phosphorylate histone H2AX (γ-H2AX) (2). γ-H2AX recruit the MRN complex, which is responsible for processing of DNA ends before ligation (3). Finally, DNA-PK recruit DNA ligase IV to seal the nick (4) and repair the break (5). In HR, the DSB is initially recognized by ATM and MRN (6). Active ATM result in phosphorylation of H2AX (γ-H2AX), (7). The 5’ strands are resected by MRN, producing long 3’ single stranded DNA tails (7), which will serve as a substrate for assembly of RAD52 epistasis group member proteins, RPA and BRCA1/2 (8). This complex searches the genome for DNA sequence homology, that is then used for strand invasion (9). Branch migration of joint DNA molecules, DNA synthesis by DNA polymeraseβ (not shown) (10). Finally, ligation (ligaseIV, not shown) and resolution of the junctions between the chromosomes (11). The figure is modified from Van den et al. 5 and Barnes et al. 7
3.5 The switch from survival response to cell death after IR-induced DNA DSBs

In addition to cell-cycle arrest, DNA DSBs may also induce programmed cell death, apoptosis, in multicellular organisms and thus eliminating cells in which damage is beyond repair capacity. This can be viewed as an alternative strategy for preventing the propagation of mutated chromosomes. Like the checkpoint response, this response to DNA damage relies on a form of signal transduction. The DNA DSB generates a signal that is ultimately received by regulators of the apoptosis pathway. However, some tumor cells retain the ability to escape apoptosis after severe irreparable DSBs and thus undergo non-apoptotic types of cell death, such as mitotic catastrophe.

3.5.1 Cell death by apoptosis

Apoptotic cell death is regarded as one of the major cell death forms after exposure to IR. Morphologically, apoptosis is characterized by condensation of nuclear chromatin, by blebbing of the nuclear and cytoplasmic membranes, and finally by fragmentation of the nuclear structures, leading to the formation of membrane-bound apoptotic bodies.

Biochemically, induction of apoptotic cell death proceeds by mitochondrially-mediated processes after DNA DSBs. Permeabilisation of the mitochondrial membrane results in the release of pro-apoptotic proteins, such as cytochrome c, is regulated primarily by the balance of anti-apoptotic members of the B-cell CLL/lymphoma 2 (BCL2) family of proteins. Remarkably, once released, cytochrome c binds to apoptotic protease activator factor 1 (APAF1). APAF1 induces the formation of a heptameric complex, the 'apoptosome', the principal regulator of a cascade of cysteine proteases (caspases) that ultimately dismantle the cell. However, in the case of nuclear stresses, such as IR-induced DNA DSBs, eventually result in the nucleus that, once in the cytosol, interact with the mitochondrial outer membrane and stimulates the release of pro-apoptotic factors.
An important factor in DSB-specific apoptosis is the TP53 protein (fig. 7).\textsuperscript{151,152,153} As mentioned earlier, TP53 can activate genes encoding proteins involved in cell-cycle arrest and repair, yet it is also capable of activating genes that induce apoptosis. In the absence of DNA DSBs, this highly pleiotropic protein is maintained at low steady-state levels. After DSB formation, TP53 rapidly becomes post-translationally modified and its level increases to allow it to regulate the expression of its effector genes.\textsuperscript{154} As explained earlier, the cellular concentration of TP53 is largely controlled by the MDM2 protein, which binds to TP53 and inhibits its activity.\textsuperscript{58,155}

Intriguingly, TP53 also posses the ability to activate the transcription of both “pro-survival” as well as pro-apoptotic genes.\textsuperscript{156,157} An explanation for this apparent contradiction is that the extent of the DNA DSBs regulates the outcome, with low levels of damage inducing a cell-cycle arrest, allowing the lesion to be repaired, and more extensive damage inducing apoptosis. In agreement with this idea, the level to which TP53 is induced seems to play a role in the choice between cell-cycle arrest and death, with apoptosis correlating with higher levels of TP53 induction.\textsuperscript{156,157,158,159} This may represent a differential ability of TP53 to efficiently trans-activate target genes, with some genes being maximally induced following relative modest TP53 accumulation, whereas others are only induced in response to a more pronounced elevation.\textsuperscript{158} Thus, under conditions of modest genomic damage, TP53 promotes
transcription and activation of genes that induce cell-cycle arrest (e.g. CDKN1A), halt cell growth and allow DNA repair to initiate. Whereas transcription of TP53-dependent genes that initiate an apoptotic cascade, such as BH3 interacting domain death protein (BID) and BCL2-associated X protein (BAX) are only required when the damage is beyond repair. Activated BID or BAX proteins thus translocate to the mitochondria, where they induces permeabilisation of the membrane by forming complexes with BCL2 proteins which lead to cytochrome c release\textsuperscript{160,161}, resulting in apoptotic death (fig. 7). This indicates that the presence above a threshold level of TP53 determines cellular fate.

In conclusion, the diverse roles of TP53 in the IR-induced DSBs response include the regulation of transcriptional programme required for efficient cell-cycle arrests and repair, as well as the triggering of apoptosis. However, TP53 is mutated in more than 50% of human cancers.\textsuperscript{162} Thus, one of the characteristic features of TP53 mutated cancer cells in conjunction with DNA DSBs appears to be resistance to apoptosis.

\subsection*{3.5.2 Cell death by mitotic catastrophe}

During recent years, the term 'mitotic catastrophe' has been widely used to describe a form of death affecting mammalian cells.\textsuperscript{163,164} Accordingly, authors view mitotic catastrophe as a type of cell death that result from aberrant mitosis.\textsuperscript{165,166,167} Such mitosis has been reported to result from a combination of deficient cell cycle checkpoints, in particular the G\textsubscript{2}/M checkpoint and the spindle assembly checkpoint.\textsuperscript{166,168,169} Loss of checkpoint functions leads to chromosome missegregation, thus inhibition of cell division, and the formation of large nonviable cells with several micronuclei (fig. 8).\textsuperscript{170,171,172}

Micronucleated cells that arise from mitotic catastrophe may be distinguished from apoptotic cells by their morphology.\textsuperscript{166,170,173,174} Although apoptotic cells may also have fragmented nuclei, they are characterized by shrunken cytoplasm and condensed chromatin, whereas cells undergoing mitotic catastrophe are large and contain uncondensed chromosomes.\textsuperscript{166,173,169} In contrast to apoptosis, it also appears that TP53 is not involved in mitotic catastrophe.\textsuperscript{168,175}
Consequently, loss of checkpoint functions in most cancer cells has been reported to be associated with their sensitivity to IR treatment.\textsuperscript{9,174,176,177} Suggesting that mitotic catastrophe may be a clinically important modality of cell death, because IR induced DNA damage (DSBs) which normally activates the DNA damage checkpoints, fails to do so in some cancer cells.\textsuperscript{176,177} Indeed, many clinical studies have demonstrated micronuclei, indicative of mitotic catastrophe in patient’s tumor samples in vivo. The incidence of micronuclei was shown to increase as a result of radiation treatment.\textsuperscript{171,178,179} Furthermore, an increase in the frequency of micronucleated tumor cells after radiotherapy was suggested to be a positive prognostic marker of treatment response.\textsuperscript{171,179,180,181}

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{Figure8.png}
\caption{Immunofluorescence analysis of a typical IR-induced micronucleated phenotype in TP53-deficient tumor cells.}
\end{figure}

untreated control cells labeled with an anti-\(\alpha\)-tubulin antibody (green, for microtubules) and propidium iodide (red, nucleus) showing a typical mitotic bipolar spindle pole in one cell (A). Irradiated (10Gy, X-rays) tumor cells, fixed and stained after 24h incubation, show micronucleated phenotype (arrow heads) indicating mitotic catastrophe (B). The figure was obtained from Sato et al.\textsuperscript{174}
3.6 Perspectives

Over the past few years, there has been much progress in our understanding of how cells detect, signal the presence of, and repair DNA DSBs, and we are beginning to understand how defect in these events are associated with carcinogenesis in humans. However, there is still much to learn. Major goals for future research will be to characterize DNA DSB responses in greater molecular detail and to identify further components of these pathways. The main key issue will be to understand how the cell coordinates the activities of the multiple systems that respond to DNA DSBs and how the relative importance of these different pathways is modulated during the cell cycle and in different cell types. Another key issue will be ascertaining how DNA DSB repair and signaling occur in the context of chromatin.

Progress in the above areas will be achieved by combining clinical knowledge with information gleaned from experiments in model organisms. A key long-term goal for this work will be to understand how DSB response pathways protect against carcinogenesis and how somatic or inherited deficiencies in these events may lead to carcinogenesis in humans. It also seems likely that this increased knowledge will lead to more effective treatment for cancer. For instance, genotyping or phenotyping individual cancers or patients for DNA DSB response pathways may lead to better predictions of how they will respond to radiotherapy and certain chemotherapies that aim to generate insupportable levels of DNA DSBs in the tumor. Moreover, increased knowledge of DSB response pathway trigger the novel anticancer drugs that target proteins involved in DSB responses in order to bring about more effective and more selective killing of cancer cells.
4. Aims of the present study

Production of DNA damage is the basis of cancer treatments, such as radiotherapy. The limitation of the treatment dose tends to be how well the tumor and normal cell within the body can tolerate the therapy. In order to improve the effectiveness of treatments, it is important to understand how cells respond to and repair IR-induced DNA damage. Of particular importance is the measurement of repair of IR-induced DNA DSBs, because these lesions, if unrepaired, lead to cell death. Recently it has been shown that histone H2AX becomes phosphorylated (termed γ-H2AX) immediately after ionizing radiation treatment and is believed to recruit DNA repair factors to sites of the DNA DSBs. In association with DSB repair, it has been reasoned that the kinetics of formation and loss of γ-H2AX foci may be related to the efficiency of the DNA breaks repair. Although image analysis of γ-H2AX foci containing thousands of γ-H2AX molecules are found at each DSB, making it possible to detect a single break within a nuclease. The main purpose of this project was to determine the cell cycle specific phosphorylation and focus formation of γ-H2AX after IR treatment of cancer cells. We also wanted to assess the role of TP53 in γ-H2AX induction, as well as in the repair, presumably reflected in the disappearance of γ-H2AX. Another question was whether repair is connected to cell cycle arrest and apoptosis.
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6. Manuscript
CELL CYCLE-DEPENDENT PHOSPHORYLATION AND FOCUS-FORMATION OF HISTONE H2AX IN X-IRRADIATED B-LYMPHOCYTE CELL LINES

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Short title: PHOSPHORYLATION OF H2AX IN B-CELL LINES AFTER X-IRRADIATION

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Abbreviations: ATM; ataxia telangiectasia mutated, ATR; ATM and RAD3-related, DSBs; DNA double-strand breaks, DNA-PKCS; DNA-dependent protein kinase catalytic subunit, HR; homologous recombination, IR; ionizing radiation, NHEJ; non-homologous end joining

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We have studied phosphorylation and focus-formation of H2AX in the human malignant B-lymphocyte cell lines Reh and U698 after ionizing radiation (IR), as well as the disappearance with time post-IR. The amount of γ-H2AX (by flow cytometry) and the number of foci (by confocal laser scanning microscopy after sorting) were higher in unirradiated cells in S and G2/M, compared to those in G1. At short times after IR (10-30 minutes), there was a dose-dependent, but not linear, increase in the amount of γ-H2AX, as well as in the number of foci, in all cell cycle phases. However, the increase at short times post-IR was most pronounced in the G1 cells, which lead to a higher amount of γ-H2AX in G1 compared to S at the higher doses (≥4Gy). Since G1 cells contain less DNA than cells in S and G2/M, this means that the amount of IR induced phosphorylation of H2AX in G1 is several-fold higher per double-strand break compared to the other phases. The IR-induced γ-H2AX foci (diameter) were significantly (p<0.001) larger than the foci in unirradiated S and G2/M cells. The decline in γ-H2AX content with time after IR was similar in the two cell lines, even though only Reh cells have an intact TP53 response, become apoptotic, and arrest in G1 after higher doses of IR. After lower doses of IR (≤1 Gy), the γ-H2AX levels returned to the levels observed in unirradiated cells within 24 hours, in agreement with the resumption of cell proliferation under those conditions. The amounts of γ-H2AX after higher doses IR (≥4Gy) also declined with time, but reached plateau levels well above those observed in unirradiated cells, under conditions where the Reh cells eventually became apoptotic and U698 cells were arrested in G2/M, respectively. The apoptotic (Reh) cells were negative for γ-H2AX. Our results suggest that there is no direct coupling between repair and TP53 status, cell cycle arrest and induction of apoptosis.

Key words: B-lymphocytes; ionizing radiation; γ-H2AX; DNA repair
INTRODUCTION

Cancer is often treated by ionizing radiation (IR). Although B-cell non-Hodgkin’s lymphomas are mostly treated by chemotherapy, some localized B-cell lymphomas are also given IR additionally.\(^1\) The response in malignant B-cells, however, is very heterogeneous, which may indicate that differences in the molecular biology caused by genetic aberrations in B-cell tumors have an impact on the outcome of the therapy.\(^2,3,4\) It is implied that treatment strategies for lymphomas based on IR could be improved with a better knowledge of the molecular mechanisms underlying the differences in radiosensitivity.\(^5\)

Exposure of the cellular DNA to IR inflicts various types of damage.\(^6\) It is well established that the induction of DNA double-strand breaks (DSBs) represents the principal lesion that, if unrepaired, may lead to genomic instability, malignant cell transformation and subsequently result in cell death.\(^7,8\) Mammalian cells have therefore evolved specialized and redundant molecular mechanisms to efficiently detect IR-induced DSBs and effect its repair. The two major DSB repair pathways are non-homologous end joining (NHEJ) and homologous recombination (HR).\(^9,10\) NHEJ has been regarded as the major mechanism for DSB repair in mammalian cells.\(^9\) This repair pathway predominates in G\(_1\) cells, but also operates in other phases of the cell cycle and joins together the two broken DNA ends independent of homology.\(^11\) In contrast, HR promotes DSB repair by using an extended, undamaged homologous sequence as template.\(^10\) Thus, HR is thought to be particularly important for DSB repair in S and G\(_2\) cells where a sister chromatid is available to as a template.\(^11,12,13\)

Phosphorylation of core histones has been proposed to be of crucial importance in facilitating the access of repair proteins to the DNA break site.\(^14\) The only chromatin modification in response to IR-induced DNA DSBs is the immediate and massive phosphorylation of histone protein H2AX in megabase chromatin regions adjacent to the breaks, yielding a specific modified form termed γ-H2AX.\(^15,16\) H2AX is a member of the histone H2A family, and its levels vary from 2-25% of the mammalian histone H2A pool depending on the cell line or tissue examined.\(^17\) Phosphorylation of H2AX is mediated by the phosphatidylinositol 3-kinase-like protein kinases ATM (ataxia telangiectasia mutated), ATR (ATM and RAD3-related), and DNA-PK\(_{CS}\) (DNA-dependent protein kinase catalytic subunit).\(^18,19,20\) These kinases are activated in response to DNA DSBs caused by IR, stalled DNA-replication forks and /or DNA damaging agents.\(^21,22\) Rogakou
et al.\textsuperscript{23} have reported a rapid $\gamma$-H2AX formation, with a half maximal amounts reached by 1-3 minutes postirradiation, and maximal amounts reached by 10-30 minutes.

It has been generally established that $\gamma$-H2AX is required for efficient accumulation of several cell cycle checkpoint and DNA repair proteins near the DSB site, forming damage induced foci.\textsuperscript{24,25,26,27,28} The frequency of microscopically visible foci per nucleus, thus, is considered to reflect the incidence of DSBs.\textsuperscript{29} Moreover, the identification of DSB-repair foci proteins suggest that the $\gamma$-H2AX plays an important role in DSBs induced in all cell cycle phases.\textsuperscript{30,31} It has been shown that H2AX-deficient cells are hyper sensitive to IR and show genomic instability, DSB repair defects, and DNA damage checkpoint dysfunction\textsuperscript{32,33}, confirming an essential role of $\gamma$-H2AX in the regulation of DNA integrity.\textsuperscript{34,35,36} Many immunofluorescence based studies have exploited the sensitivity of $\gamma$-H2AX foci to address questions about the role, timing and distribution of DSBs. Several groups have reported that the amount of H2AX phosphorylation after IR-induced DNA DSBs increased linearly with IR dose.\textsuperscript{30,37,38,39,40} In addition to DSB repair, amount of $\gamma$-H2AX has been reported to increase in apoptotic cells, suggesting that H2AX phosphorylation is an early chromosome modification that is followed by apoptotic DNA fragmentation\textsuperscript{41}, and constitutes an important step in the course of mammalian apoptosis.\textsuperscript{42,43}

Thus, development of methods to predict tumor cell response to DSBs represents an important approach to improving response to radiation therapy. Of particular importance is the integration of the measurements established for estimating repair kinetics and apoptosis after IR-induced DNA DSBs. In this work we have used flow cytometry and confocal laser scanning microscopy to study the phosphorylation and focus-formation of H2AX immediately after different doses of IR in the human B-lymphocyte cell lines Reh and U698. The kinetics of decay of this initial phosphorylation was also assessed.
MATERIALS AND METHODS

Cell culture and irradiation

The human malignant B-cell lymphocyte cell lines Reh\textsuperscript{44} and U698\textsuperscript{45} were used. The cells carry wild-type \textit{TP53} alleles.\textsuperscript{46} However, while Reh cells have two \textit{TP53} alleles and show an increase in \textit{CDKN1A} mRNA and CDKN1A protein expression after X irradiation\textsuperscript{47}, U698 cells have only one \textit{TP53} allele (unpublished results) and show no induction of either \textit{CDKN1A} mRNA or CDKN1A after irradiation.\textsuperscript{47} Thus, it is suggested that the TP53 pathway is not functional in U698 cells.\textsuperscript{47} Additionally, U698 cells do not express RB1.\textsuperscript{46} The growth characteristics of the cell lines and phenotypic changes after irradiation are described elsewhere.\textsuperscript{4}

The cells were grown as single cell suspension in RPMI 1640 supplemented with 10\% fetal calf serum, 1\% L-glutamine and 1\% penicillin-streptomycin (all from PAA laboratories GmbH, Pasching, Austria), and kept at 37\textdegree C in 5\% CO\textsubscript{2} atmosphere with 95\% humidification. The cells were seeded out at a density of 3.0 \times 10^6 cells in 10ml medium 24 hours before X-irradiation. In all experiments, the cell lines were confirmed to be free from mycoplasma before use.

Cells were irradiated in 25cm\textsuperscript{2} flasks at room temperature with 200KeV X-rays (Simens, Munich, Germany; dose rate at 3.3Gy/minute, 0.5mm copper filter) at doses of 0 (control), 0.2, 0.5, 1, 4, 10, and 20 Gy. Cells were harvested at 10min, 30min, 1, 2, 4, 10, and 24 hours after irradiation. Samples were then washed once with phosphate buffered saline (PBS), fixed in 70\% ethanol, and stored at -20\textdegree C until analysis for histone protein \textit{\gamma-H2AX}.

\textit{Staining for \textit{\gamma-H2AX} protein}

All steps were performed at 0\textdegree C. Fixed cells (approximately 3.0 \times 10^6 cells per sample) were centrifuged and washed once in PBS. The cells were resuspended in 50\textmu l detergent buffer (0.1\% Nonidet P40, 6.5 mM Na\textsubscript{2}PO\textsubscript{4}, 1.5 mM KH\textsubscript{2}PO\textsubscript{4}, 2.7 mM KCl, 137 mM NaCl, 0.5 mM EDTA, pH 7.5) with 4\% (w/v) nonfat dried milk for blocking of non-specific binding. Thereafter, 50 \textmu l of detergent buffer with mouse monoclonal anti-histone \textit{\gamma-H2AX} antibody (Upstate, Lake placid, NY; 1/250 dilution, i.e. final dilution 1/500) was added, and incubation was continued for 30 minutes. The control samples
received no primary antibody. After washing in PBS, cell pellets were resuspended in 100µl FITC-conjugated rabbit anti-mouse secondary antibody (DAKO Cytomation, Glostrup, Denmark) diluted 1:25 in detergent buffer with 4% (w/v) dried nonfat milk, and incubated for 30 minutes. Finally, after washing with PBS, the cells were resuspended in PBS containing 5 µg/ml propidium iodide (PI; Calbiochem, La Jolla, CA) and 100 µg/ml RNase A (BD biosciences clontech, Palo Alto, CA) for DNA staining and analysis of the amount of γ-H2AX by flow cytometry. For cell sorting and subsequent confocal laser scanning microscopy analysis, 2 µg/ml Hoechst 33258 dye (Calbiochem, La Jolla, CA) for staining of DNA was used instead of PI.

Detection of apoptotic cells

All steps were performed at 0°C. Fixed cells were washed once in PBS and incubated for 30 minutes at 37°C in 50 µl terminal deoxynucleotidyl transferase (TdT) solution containing 5 units TdT, 10 µl 5x reaction buffer, 1.5 mM CoCl₂ (all in TdT kit; Roche diagnostics GmbH, Indianapolis, IN, USA), 0.1 mM dithiothreitol, distilled water and 0.5 nmol Cy5-dUTP (Amersham Pharmacia Biotech, Little Chalfont Bucks, England). The cells were thereafter washed once with PBS, centrifuged, and staining for γ-H2AX was then performed as described above. The pellet was resuspended in PBS containing 5 µg/ml PI and 100 µg/ml RNase A for DNA staining.

Flow cytometry and sorting

Cells stained for γ-H2AX and DNA, and in some cases also for apoptosis, were analyzed in a FACSCalibur flow cytometer (Becton Dickinson, San Jose, CA) equipped with one argon (488nm) and one diode (635nm) laser. PI (>630nm, DNA staining) and FITC fluorescence (510-540nm, γ-H2AX staining) were measured with excitation at 488nm, and Cy5 fluorescence (650-670 nm, apoptosis) with excitation at 635nm. At least 10 000 cells were measured per sample. Doublets and cell aggregates were discriminated from the analysis by gating around the singlet population in the PI fluorescence pulse width versus pulse area dual parameter cytogram (not shown in the figures). PI fluorescence pulse area was used as a measure of cellular DNA content. The median FITC fluorescence intensity (γ-H2AX) was estimated in each cell cycle phase. The corresponding fluorescence intensities measured in the control samples were subtracted to
obtain γ-H2AX specific fluorescence. Intensities were calibrated with alignment beads (488nm; Molecular probes, Oregon, USA) prior to each experiment such that the FITC fluorescence intensity measured in different experiments could be compared.

To obtain anti-γ-H2AX stained cells from different phases of the cell cycle, cells were sorted based on Hoechst 33258 intensity into G1, S, and G2/M phase populations using a DIVA flow cytometer (Becton Dickinson, San Jose, CA) equipped with an argon laser (488nm; for detection of anti-γ-H2AX staining) and one UV laser (351/357nm; Hoechst 33258 for DNA staining; emission filter). Hoechst 33258 fluorescence pulse area versus width was used to discriminate doublets and aggregates, so that these could be excluded during the sorting. γ-H2AX-negative cells (necrotic or apoptotic; see “Results”) were also excluded during the sorting. At least 10 000 cells were sorted per sample. 10 µl anti-fade Vectashield mounting medium (Vector laboratories, Burlingham, USA) was directly added to the sorted cells to prevent photobleaching of FITC.

*Laser scanning confocal microscopy for the imaging of γ-H2AX foci*

Sorted cells in anti-fade solution were imaged in a Zeiss LSM 510 META confocal laser scanning microscope (Carl Zeiss, Göttingen, Germany) equipped with an argon laser for excitation at 488nm. Images were acquired using a 63x objective. Optical sections throughout the thickness of the cell were captured at 0.5µm intervals and combined in a maximum projection by LSM 510 Expert mode software (Carl Zeiss, Jena, Germany) so that all the visible foci were recorded. For quantitative analysis, γ-H2AX foci were counted by visual scoring of 10 G1, S, and G2/M cell cycle phase cells for each IR dose. Thus, the average number of foci per cell in each cell cycle phase was estimated as the mean number of foci scored in these 10 cells by manually analyzing their confocal image stacks. A setting that excluded background speckles was used as a standard for foci quantification in all the cells selected for analyses. Same cells were also analyzed for focus diameter by the Analysis software (Soft imaging system GmbH, Munster, Germany). For 3D view of γ-H2AX foci distribution, optical sections were captured as 64 series of projections for 3D display, with Y as the tuning axis. The computed 3D sequence (by LSM 510 Expert Mode software) can be animated or viewed as gallery at attached CD (see supplementary material).
RESULTS

Cell cycle-specific phosphorylation of H2AX in control and irradiated cells

The content of γ-H2AX in the different phases of the cell cycle was measured by flow cytometry (Fig.1). The amount of γ-H2AX in unirradiated control cells was much higher in the G2/M, and particularly in the S phase cells, compared to cells in G1, which was directly evident from the "horse-shoe" like (with opening down) cytograms of control cells (Fig.1, pre-IR (control)). After subtraction of the fluorescence intensities of the cells stained in the absence of the primary antibody (Fig.1, staining control), the γ-H2AX-specific intensities were 15 and 36 for the G1 cells, 66 and 105 for the S phase cells, and 79 and 101 for the G2/M cells, respectively, for Reh and U698 cells. The amount of γ-H2AX increased at short times after irradiation (Fig.1, 30 minutes). The latter cytograms also had a horse-shoe like shape, but were inverted, i.e. the opening was up, compared to the cytograms of control cells, showing directly that the increase in γ-H2AX was largest in G1, followed by G2/M and S. (The cytograms of staining controls were similar for control and irradiated cells.) Some γ-H2AX-negative cells were observed at short times (10minutes-2hours) after irradiation in both cell lines (Fig.1, 30 minutes). The fraction of γ-H2AX-negative cells at short times post-IR increased with radiation dose. This subpopulation of γ-H2AX-negative cells disappeared with time (Fig.1, 4 hours), suggesting that these cells were necrotic.

A γ-H2AX-negative subpopulation of cells was also observed in cultures of Reh cells irradiated with ≥4Gy at 24 hours post-IR (Fig.1, 24 hours, left panel). Reh cells, but not U698 cells, become apoptotic after higher doses of IR (≥4Gy). We therefore combined staining for γ-H2AX with staining for apoptosis by the TdT procedure to further define the different subpopulations (Fig.2). Figure 2A shows that, as expected, a large fraction of Reh cells were apoptotic, this cytogram was used to define the region containing apoptotic cells as shown by the dotted areas in Figures 2C, D, and E. The γ-H2AX-negative cells were apoptotic (Fig.2D, gate shown in Fig.2B). However, whereas almost all γ-H2AX-positive cells were non-apoptotic, a small subpopulation of G2/M cells was found to be apoptotic (Fig.2E). Gating on the subpopulation of G2/M cells staining brightly for γ-H2AX (Fig.2B) revealed that the majority of these were apoptotic (Fig.2C), accounting for the apoptotic cells in Fig.2C.
Cell cycle-resolved number and size of the foci before and immediately after irradiation were investigated by confocal laser scanning microscopy of G₁, S, and G₂/M cells sorted by flow cytometry (see “Materials and Methods”; sort gates not shown). One (median) confocal slice (of the stack of images of each cell) is shown in Figure 3 for both cell lines in the different cell cycle phases after different radiation doses. The complete set of slices for one cell in each cell cycle phase after the different doses can be found on the attached CD (see supplementary material). The CD also contains three-dimensional reconstruction (3D) of the same cells.

**Phosphorylation and focus-formation of H2AX immediately after irradiation**

The dose-response of the phosphorylation of H2AX was investigated by flow cytometry and confocal laser scanning microscopy as shown in Figures 1 and 3 at a short time after irradiation (Fig.4, 30 minutes; similar results were obtained after 10 minutes and 1 hour, see Fig.5). The amount of γ-H2AX increased monotonously with dose, but not in a linear fashion above 1 Gy (Fig.4, upper panels). It could even appear that plateau levels were reached above 10Gy, particularly in U698 cells.

Control and irradiated cells were sorted based on DNA content to study the number and size of the γ-H2AX foci. The middle panels of Figure 4 show that the number of foci increased monotonously with dose, but again, the increase was not linearly related to dose. Unirradiated G₁ cells had less than 1 focus per cell, which increased to approximately 15 after 1Gy and 25 after 4Gy. There was a much higher number of foci in control S and G₂/M cells compared to the ones in G₁. Although some late S phase cells may have been included in the G₂/M sorted fraction, the relatively tight standard deviations show that the (control) G₂/M cells have a much higher number of foci than cells in G₁. However, control S cells had a higher number of foci than the corresponding G₂/M cells. This situation was reversed after 4 Gy, when the G₁ cells had a higher or equal number of foci than the G₂/M cells, which showed a higher number of foci than the S phase cells.

The non-linear increase in the number of foci with increased radiation dose could have been an artifact due to an apparent fusion of foci due to limited resolution in the microscope. However, although the size of the foci increased in all cell cycle phases increased slightly after irradiation with 0.2Gy (Fig.4, lower panels), there was no further increase in focus size with increasing dose, arguing against this hypothesis.
The disappearance of γ-H2AX with time

Figure 5 shows the time-dependency of the content of γ-H2AX in the different phases of the cell cycle after irradiation with different doses. There was an initial increase in γ-H2AX content, which reached a maximum after 10 minutes to 1 hour. After this initial increase, the γ-H2AX-associated fluorescence decreased and returned to control values after low doses of IR (≤1Gy). The amount of γ-H2AX decreased also after the higher doses of IR, but did not approach the levels seen in control cells.

DISCUSSION

We have studied cell cycle-resolved phosphorylation and focus-formation of H2AX in two human malignant B-lymphocyte cell lines after ionizing radiation. The amount of γ-H2AX and the number of foci were higher in unirradiated cells in S and G2/M, compared to those in G1. At short times after IR there was a dose-dependent, but not linear, increase in the amount of γ-H2AX, as well as in the number of foci, which was most pronounced in the G1 cells. The repair kinetics were similar in the two cell lines, even though only Reh cells have an intact TP53 response, become apoptotic, and arrest in G1 after higher doses of IR. The amounts of γ-H2AX after higher doses of IR (≥4Gy) declined with time, but reached plateau levels well above those observed in unirradiated cells, under conditions where the Reh cells eventually became apoptotic and U698 cells were arrested in G2/M, respectively. The results suggest that there is no direct coupling between repair and TP53 status, cell cycle arrest and induction of apoptosis.

The formation of foci consisting of phosphorylated H2AX after IR have been studied by both microscopy and flow cytometry.\textsuperscript{29,31,37,38,48} It has been concluded from most of these studies that the number of foci and the amount of γ-H2AX are directly related to the number of IR-induced double strand breaks. However, it has been noted that there are foci in unirradiated S phase cells, and that the content of γ-H2AX is slightly higher in S phase cells compared to cells in G1 and G2/M phases of the cell cycle.\textsuperscript{20,30,49} The foci in unirradiated S phase cells have been described as smaller and possible to distinguish from the radiation-induced foci.\textsuperscript{30} Our results show that there is a significant amount of γ-H2AX, clustered in foci, in control S and G2/M phase cells, at least in the
human malignant B lymphocyte cell lines studied here. It has been assumed that the \( \gamma \)-H2AX observed in undamaged cells is associated with DNA replication.\(^{20,21,49}\) This is not in agreement with our finding that (control) G\(_2\)/M cells have almost as much \( \gamma \)-H2AX and almost as many foci as S cells. Hence, \( \gamma \)-H2AX may have a more complex role than simply being associated with unwound and denatured DNA in the vicinity of replication forks.

The amount of \( \gamma \)-H2AX, as well as the size and number of the foci, increased monotonously with radiation dose immediately after irradiation (Figs. 1, 3, 4). In contrast to reported observations\(^{30,37,38,39,40}\), our results showed that this increase of \( \gamma \)-H2AX was not linearly related to IR dose. However, there was a dependency on cell cycle phase, where the \( \gamma \)-H2AX content in G\(_1\) cells increased considerably more than in the G\(_2\)/M and S cells. Considering that the cells in G\(_1\) have less DNA than the cells in S and G\(_2\)/M, we conclude that the degree of phosphorylation of H2AX per DSB is approximately two-fold higher in G\(_1\) than in the rest of the cell cycle. It is thus indicated that the H2AX-kinase activity (after radiation) is higher in G\(_1\) than in S and G\(_2\)/M. This increased activity could be caused by cell cycle-specific activation of e.g. DNA-PK\(_{CS}\), since this enzyme is involved in detection of DSBs and initiation of repair by NHEJ in G\(_1\) cells.\(^{19,22}\) TP53 does not seem to be involved upstream of the phosphorylation of H2AX, as the latter proceeds as well in U698 cells with defect in the TP53 pathway.\(^{47,50}\)

The presence of \( \gamma \)-H2AX foci has been considered to reflect the number of DSBs.\(^{23,29}\) However, recently published studies show that the DSBs are apparently repaired before the \( \gamma \)-H2AX levels decline.\(^{37,51}\) After low doses of IR (\( \leq 1 \) Gy), we observed that the \( \gamma \)-H2AX levels declined to the levels observed in control cells approximately at the time when the cells resumed proliferation (fig.5). Recently, Olive et al.\(^{39}\) have examined the kinetics of \( \gamma \)-H2AX after irradiation of 10 cell lines, suggesting that the rate of loss of \( \gamma \)-H2AX is associated with a greater ability to survive after exposure to low IR doses.\(^{38,39}\) Additionally, \( \gamma \)-H2AX foci at the site of DNA strand break is thought to be required for the retention of repair proteins in the vicinity of the break.\(^{31}\) Several DNA repair proteins involved in the DNA damage response have been shown to subsequently colocalize with \( \gamma \)-H2AX at the sites of the strand breaks in response to IR.\(^{25,26,28,31}\) Some of these DNA repair proteins physically interact with \( \gamma \)-H2AX to form these nuclear foci.\(^{25,26}\) These findings indicate that \( \gamma \)-H2AX is required for the recognition and repair of DNA DSBs\(^{31}\), and that the loss of these foci might related to the efficiency of repair.\(^{37}\) On the other hand, the \( \gamma \)-H2AX levels did not return to control values after
higher doses of IR (≥4 Gy, fig.5). Under the latter conditions, the cells are either becoming apoptotic (Reh) or are permanently arrested in G2/M.4 Our results may suggest that even though γ-H2AX may not be a direct measure of DSBs, the γ-H2AX levels must decline to pre-IR values before proliferation can be resumed.

The apoptotic (Reh) cells were negative for γ-H2AX (fig.2). This result is not in agreement with previously reported results30,41,42,52, as the latter authors found that the apoptotic cells had higher levels of γ-H2AX. However, both the cell types and the treatments were different in their study and ours. Additionally, we identified a subpopulation of G2/M cells with increased γ-H2AX content, probably representing early apoptotic cells. In contrast to Reh cells, U698 cells have defects in the TP53 pathway.47 Cells with loss of wild-type TP53 function have a reduced ability to become apoptotic, accompanied by higher radiation resistance.53 Thus, U698 were only arrested in the G2/M phases when irradiated with higher doses at 24 hours post-IR4, and were all γ-H2AX positive (fig.1). Recent studies have shown that γ-H2AX is not important for the TP53 response to DNA DSB damage50, which indicates that phosphorylation of H2AX function largely in distinct pathways of TP53 in DNA DSB damage responses. In conclusion, these results together with repair kinetics suggest that there is no direct coupling between disappearance of γ-H2AX and TP53 status, cell cycle arrest and induction of apoptosis after IR. Thus measurement of DNA DSB induction and repair by γ-H2AX expression in human cancer cells could be linked to clinical effectiveness of radiotherapy with a view to explaining the outcome, predicting response, and designing treatments that may change the clinical results.

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REFERENCES


FIGURE LEGENDS

FIGURE 1. Flow cytometry analysis of the content of $\gamma$-H2AX and DNA before and after irradiation in Reh (left) and U698 (right) cells. The cell lines were exposed to 4Gy x-rays and fixed at various times, as indicated, and subsequently stained with anti-$\gamma$-H2AX (vertical axis, log scale) and propidium iodide to detect total DNA (horizontal axis, linear scale). The insets show the corresponding DNA histograms. The staining control (second panels) represent cells stained in the absence of primary antibody.

FIGURE 2. Apoptotic Reh cells induced by the higher doses of IR ($\geq$ 4Gy) after 24h, were negative for $\gamma$-H2AX. Reh cells were exposed to 4Gy x-rays, fixed 24h post-IR and stained for both $\gamma$-H2AX and apoptosis (TdT), as described in materials and methods. Cells were stained with propidium iodide for DNA content. Cytogram A show all cells, which was then used to define the region containing apoptotic cells as shown by the dotted area in C, D, and E. The $\gamma$-H2AX-negative cells (lower gate, B) were apoptotic (D), $\gamma$-H2AX-positive cells (upper gate, B) were non-apoptotic (E) with exception of G2/M cells among the $\gamma$-H2AX positive (subpopulation gated, B) which was found to be apoptotic cells (C).

FIGURE 3. Cell cycle specific $\gamma$-H2AX foci formation before and after irradiation in Reh (left) and U698 (right) cells. Both cell lines were irradiated with various doses, as indicated, fixed 30min post-IR and subsequently stained with anti- $\gamma$-H2AX and Hoechst 33258 for DNA content. Cells were sorted by flow cytometry on the basis of their DNA content and subsequently examined under a laser scanning fluorescence microscope, as described in materials and methods.
**FIGURE 4.** Analysis of dose-response of γ-H2AX formation sampled at 30min post irradiation in Reh (left) and U698 (right) cells. Relative γ-H2AX levels (flow cytometry; upper panels) were obtained by subtracting the staining control (pre-IR). Error bars represent standard error of the mean of three independent experiments. The middle panels show the cell cycle dependency of the increase in mean focus number in control and irradiated cells. The lower panels show the corresponding mean focus diameters. Error bars in the middle and lower panels show the standard deviation of 10 cells per cell cycle phase.

**FIGURE 5.** Cell cycle dependency of the disappearance of γ-H2AX. The plots show the time-dependency of the γ-H2AX content (measured by flow cytometry) in the different cell cycle phases after irradiation with various doses, as indicated. Apoptotic (Reh) cells were excluded from the analysis. Plotted values represent the relative γ-H2AX obtained by subtracting the staining control. Error bars represent standard error of the mean from three independent experiments.
Figure 1

Reh

U698

γ-H2AX

pre-IR

Staining control (pre-IR)

30 min post-IR

4 h post-IR

24 h post-IR

DNA content

Figure 1
Figure 2
Figure 3
γ-H2AX (arbitrary units)

Number of foci/cell

Focus diameter (µm)

Radiation dose (Gy)

Figure 4.
Figure 5

Reh

U698

Amount of γ-H2AX (arbitrary units)

Time (hours)

G1

S

G2/M

0 5 10 15 20 25 30

0 5 10 15 20 25 30

0 5 10 15 20 25 30

0 0.5 1.0 1.5 2.0

0 0.5 1.0 1.5 2.0

0 0.5 1.0 1.5 2.0

0.2Gy 0.5Gy 1Gy 4Gy 10Gy 20Gy

0.2Gy 0.5Gy 1Gy 4Gy 10Gy 20Gy

0.2Gy 0.5Gy 1Gy 4Gy 10Gy 20Gy

0.2Gy 0.5Gy 1Gy 4Gy 10Gy 20Gy

0.2Gy 0.5Gy 1Gy 4Gy 10Gy 20Gy

0.2Gy 0.5Gy 1Gy 4Gy 10Gy 20Gy

G2 /M

S

G2 /M

0 5 10 15 20 25 30

0 5 10 15 20 25 30

0 5 10 15 20 25 30

0 0.5 1.0 1.5 2.0

0 0.5 1.0 1.5 2.0

0 0.5 1.0 1.5 2.0

0.2Gy 0.5Gy 1Gy 4Gy 10Gy 20Gy

0.2Gy 0.5Gy 1Gy 4Gy 10Gy 20Gy

0.2Gy 0.5Gy 1Gy 4Gy 10Gy 20Gy

0.2Gy 0.5Gy 1Gy 4Gy 10Gy 20Gy

0.2Gy 0.5Gy 1Gy 4Gy 10Gy 20Gy

0.2Gy 0.5Gy 1Gy 4Gy 10Gy 20Gy

S

G2 /M

Time (hours)
7. Supplementary material

CELL CYCLE-DEPENDENT PHOSPHORYLATION AND FOCUS-FORMATION OF HISTONE H2AX IN X-IRRADIATED B-LYMPHOCYTE CELL LINES

Marwa JALAL and Trond STOKKE

Supplementary movies;

Cell cycle-resolved histone γ-H2AX foci before and after irradiation investigated by confocal laser scanning microscopy of G1, S, G2/M Reh (CD 1) and U698 (CD 2) cells. Each movie shows all the (horizontal) sections throughout the thickness of one cell in each cell cycle phase combined in a maximum projection. Three dimensional (3D) reconstructions (Y axis) from the same sections demonstrate the total distribution of γ-H2AX foci in both cell lines. The movies are presented as Quick Time (Apple) files.