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Reviews

Regulation of the cell cycle following DNA damage in normal and Ataxia telangiectasia cells

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Abstract. A proportion of the population is exposed to acute doses of ionizing radiation through medical treatment or occupational accidents, with little knowledge of the immedate effects. At the cellular level, ionizing radiation leads to the activation of a genetic program which enables the cell to increase its chances of survival and to minimize detrimental manifestations of radiation damage. Cytotoxic stress due to ionizing radiation causes genetic instability, alterations in the cell cycle, apoptosis, or necrosis. Alterations in the G1, S and G2 phases of the cell cycle coincide with improved survival and genome stability.

The main cellular factors which are activated by DNA damage and interfere with the cell cycle controls are: p53, delaying the transition through the G1-S boundary; $p21^{WAF1/CIP1}$, preventing the entrance into S-phase; proliferating cell nuclear antigen (PCNA) and replication protein A (RPA), blocking DNA replication; and the p53 variant protein p53as together with the retinoblastoma protein (Rb), with less defined functions during the G2 phase of the cell cycle. By comparing a variety of radioresistant cell lines derived from radiosensitive ataxia telangiectasia cells with the parental cells, some essential mechanisms that allow cells to gain radioresistance have been identified. The results so far emphasise the importance of an adequate delay in the transition from G2 to M and the inhibition of DNA replication in the regulation of the cell cycle after exposure to ionizing radiation.

Key words. Ataxia telangiectasia; cell cycle; radiation resistant DNA synthesis; DNA damage; DNA repair; cell cycle check point.

Introduction

The integrity of genetic information is under threat from the accumulation of genetic errors, as a result of mistakes during DNA replication and repair. Some of these mutations are incompatible with the continuation of cellular processes and cause cell death. The observation that DNA damage induced genome instability over many cell divisions after the exposure[57,68,53] raises questions about the molecular mechanisms involved in the transmission of DNA damage to chromosomal instability and increased mutation frequencies. It is conceivable that mutations in genes controlling the structural integrity of DNA could increase the risk of genetic instability and cause secondary mutations, independent of the site of the initial DNA damage. The increased genetic instability seen in p53 mutants[7,27,63,153] as well as the suppression of recombination by topoisomerase II (for review see ref. 174) are strong indicators of such mechanisms.

Interlinked with DNA repair systems is cell cycle control, and a large number of mammalian cell systems with increased sensitivity to ionizing radiation and failure to regulate the cell cycle after DNA damage have been identified. The most thoroughly investigated are from patients suffering from ataxia telangiectasia (AT). These cells fail to arrest the cell cycle after irradiation and the induction of key factors in response to ionizing radiation is suboptimal. Attempts by different laboratories to identify the AT gene(s) by the method of DNA-mediated gene transfer has resulted in the isolation of radiation-resistant derivatives of AT cells. Radiosensitive cell lines from AT patients and their radioresistant derivatives differ in adaptation of the cell cycle and regulation of DNA replication after cytotoxic stress, and thus represent different molecular mechanisms that optimize passage through the cell cycle in order to increase cell survival. The recent identification of the ATM gene by positional cloning[138] provides the opportunity to compare the molecular mechanisms of phenotypic AT revertants in cell cycle regulation with the function of the ATM gene product.

The key principles of the eukaryotic cell cycle

The events in the eukaryotic cell cycle can be divided into two classes. First, a cell cycle engine based on the activation and inactivation of $p34^{cdc2}$ during the passage through G1, S, G2 and M phases of the cell cycle; second, a set of events which can arrest the cell cycle engine at certain checkpoints in response to cytotoxic

damage, to ensure the completion of early events before late ones begin. The elimination of checkpoints by mutations or overexpression of regulatory functions results in an increased susceptibility to DNA-damaging agents or even cell death (for review see ref. 177). Thus, the efficacy of checkpoints in the control of the cell cycle affects the genetic stability and the survival of a cell. The possibility of competing signals will make the regulation of these transitions rather complex. In mammalian cells, as in yeast, two checkpoints have been identified: one at the transition from G1 to S phase and a second between G2 to M[54].

In higher eukaryotes, the cell cycle engine depends on at least two cyclin-dependent kinases *cdc2* and *cdk2* and on their ability to bind cyclins. Six cyclins have been characterized to date (cyclins A, B, C, D, E, and F), but specific functions have been identified for only a few of these. However, the following principles can be put forward: in the G1 phase of the cell cycle, $p34^{cdc2}$ lacks any associated cyclins and has no protein kinase activity. Under normal growth conditions, cyclins C, D, E and F accumulate in G1, bind to $p34^{cdc2}$ and activate its protein kinase activity, promoting the cells through the restriction point into S phase. During G1 the retinoblastoma (Rb) protein is hypophosphorylated and complexed with E2F, inhibiting its transcription factor activity of genes encoding enzymes required for DNA synthesis[13]. During the transition from G1 to S, the Rb protein is phosphorylated, dissociates from E2F (thereby activating the transcription factor) and forms a new complex with p107 (closely related to Rb), cyclin A and p33 (a homolog of p34)[25,144]. It is only after the passage through the G1-S transition point that the cells express cyclin A, which together with p33, control the events in S-phase[48,37].

In contrast to mammalian cells, which express a multitude of $p34^{cdc2}$-related proteins, simple eukaryotes such as yeast manage with a single *cdc2* cell regulator, which has helped to understand the cell cycle events following S-phase. After the passage through the G1-S transition point (START), yeast cells synthesize cyclin B, which binds to $p34^{cdc2}$ thereby blocking its protein kinase activity through the phosphorylation of tyrosine-15. Together with the phosphorylation of threonine-160, this inactive complex is referred to as pre-maturation promotion factor (pre-MPF). The removal of the tyrosine phosphate from $p34^{cdc2}$ during late G2 activates MPF and, through the activation of tyrosine phosphatase and inhibition of tyrosine kinase function, a spike of MPF drives the cells rapidly into mitosis. Non-replicated DNA blocks the activation of cyclin B-cdc2 complex by preventing dephosphorylation of Tyr-15 in $p34^{cdc2}$ [34]. The degradation of cyclins during M phase prepares the cells for the next cell cycle (for review see refs 64, 106, 114, 143, 55, 75).

What are the regulatory mechanisms of the cell cycle and how do they respond to ionizing radiation?

Ionizing radiation causes a wide spectrum of damage to DNA, of which double-strand breaks (dsb) are associated with lethality[10,40]. In eukaryotes dsb repair seems to utilize mechanisms that are part of the DNA recombination pathway[42,155]. A complex variety of programmed responses including activation of DNA repair, cell cycle arrest and apoptosis are invoked by ionizing radiation (for review see refs 175, 99), but the genes which integrate DNA repair with cell cycle progression remain to be identified. Gene products upregulated in the process are the transcription factors NFkB, c-Fos, c-Jun, EGR1 and p53[8,49,176,71]; in contrast, cell cycle regulatory genes such as cyclin A, cyclin B, *cdc2* and *cdc25* are downregulated[21,94,107]. The cellular stress response includes mechanisms to arrest the cell cycle in G1 and G2 as well as an inhibition of DNA synthesis[24,121,167].

G1 and the G1-S checkpoint

The checkpoint for the transition from G1 to S phase regulates the start of the replication of DNA. The initiation of DNA synthesis has been found to be extremely sensitive to single-strand lesions[173,120]. One single-strand break may inactivate the initiation of as many as a hundred replicons[50]. This control may prevent re-initiation of any unligated strands remaining from the previous S-phase. Lethal doses of ionizing radiation induce alterations in DNA metabolism in mammalian cells resulting in an increased uptake of nucleoside precursors of cells in S phase[150], unscheduled DNA synthesis in non-S phase cells (however, less efficient than after UV irradiation)[130], and a rapid rejoining of single- and double-strand breaks[89,80,81].

These findings in tissue culture experiments have been corroborated by experiments using cells taken from human tissue. Irradiation of bone marrow cells with doses between 5 and 50 Gy decreased the rate of DNA synthesis and blocked the cell cycle in its transition from G1 to S. Irradiation with only 2–3 Gy only showed a block of cell cycle progression, but had no effect on the DNA synthesis[82]. A similar decreased transition from G1 to S has been observed in extremely slowly dividing diploid human amnion cells after exposure to doses as low as 0.1 to 3 Gy, with an almost complete block in G1 phase after exposure to 3 or 10 Gy[91]. This indicates that there could be a dual response of the cell cycle to ionizing radiation depending on dose.

Following irradiation with 10 Gy, arrest in G1 coincides with an increase in p53 levels 1–2 hours after irradiation, due to an increased stability of the protein[71]. In general, cell lines which express wildtype p53 exhibit a radiation induced G1-arrest, unlike cell lines expressing mutant p53 or lacking p53 altogether[79,71]. A partial

restoration of radiation-induced G1 arrest has been observed in p53-deficient HL60 cells after transfection of the wildtype p53[115]. Fibroblasts from p53 'knockout' mice showed no G1 arrest after exposure to 2 or 4 Gy, compared to a marked G1 arrest of fibroblasts with two intact p53 genes; fibroblasts from mice with only one intact p53 gene showed an intermediate response[72]. The combination of p53 expression and G1 arrest however, does not correlate with radioresistance[149]. There is rather an indication of the reverse: bone marrow from transgenic mice with dominant mutant p53 shows increased radioresistance compared to wildtype tissue[86]. There is clear evidence that wildtype p53 can function as a transcription factor on templates containing the p53 recognition sequence and that mutant p53 inhibits this process[38,131]. Several genes have been identified that contain the p53 binding sequence, including the *gadd45*[72] and *mdm2* genes[1]. The mechanism whereby DNA-binding and transcriptional activity of p53 is regulated is only partly understood. Various experiments indicate that the interaction of as yet unknown cellular factors, or the phosphorylation of a casein kinase II site, antibody Pab421 binding or the deletion of 30 amino acids at the C terminus, activate DNA binding of p53[61].

The elevation of p53 levels after exposure to X and UV irradiation activates p53-dependent promoters[97] increasing MDM-2[126], GADD45[72] and $p21^{WAF1/CIP1}$[32] protein levels, which then influence progression through the cell cycle (see fig. 1). However, more genes have been shown to be repressed by p53 including c-*fos*, c-*jun*, *β-actin*, hsp70, interleukin 6 and p53 itself; the mechanisms of the repression are unknown[47,136]. But the induction of growth arrest after DNA damage does not exclusively depend on the activation of p53. The induction of *gadd153* after DNA damage is p53-independent and growth arrest is achieved by an unknown mechanism[39].

A link between the growth-suppressing activity of p53 and the inactivation of cyclin-dependent kinases (Cdk) has been provided by the cloning of the p21 gene (also called *WAF1/CIP1*) the transcription of which is directly activated by p53[32,52,181]. $p21^{WAF1/CIP1}$ is induced by DNA-damaging agents that trigger G1 arrest or apoptosis. However, this induction can only be observed in cells with wild-type p53, and not with mutant p53[33]. $p21^{WAF1/CIP1}$ has been shown to bind to several human cyclin-dependent kinases involved in the G1-S transition and to inhibit their kinase activity[52,29]. In cells arrested in G1 by radiation, $p21^{WAF1/CIP1}$ binds to the Cdk-cyclin E complex and inhibits its kinase activity preventing the cells from entering S-phase[29,33,181].

Preceding the G1-S checkpoint, the activity of the *Egr-1* gene is required for the transition of quiescent cells from G0 to G1[154]. Egr-1 is a nuclear phosphoprotein with a zinc finger and partial homology to the

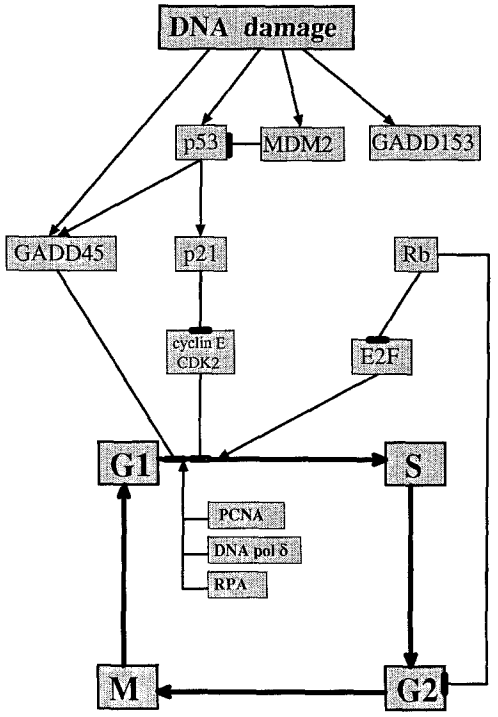

Figure 1. Schematic representation of DNA damage-induced regulation of the cell cycle. ↓ indicates stimulation, ⊥ indicates the blocking of a function. Abbreviations and explanations, see text.

Wilms' tumour suppressor gene, and functions as a transcription factor[49]. In the promoter of the *Egr-1* gene, an AP-1 recognition sequence has been found, identical to promoters of other X-ray-inducible genes including c-*jun*, platelet derived growth factor, basic fibroblast growth factor, tissue plasminogen activator and tumour necrosis factor-α[176]. Schematically, the induction of c-*fos* and c-*jun* as an immediate early response to ionizing radiation leads to the binding of the activated Jun protein in a complex with Fos (or another unknown nuclear protein) to the AP-1 sequence and to the activation of *Egr-1* and other genes. The cross-coupling of two different classes of transcription factors like NF-κB with AP1 would drastically increase the potential for the formation of protein complexes exhibiting specific biological activity[152]. Itself a transcription factor with specific DNA-binding activity, the *Egr-1* gene is an intermediate step in the X-ray modulation of gene expression and will release a further cascade of gene products[154]. In addition to AP-1-dependent activation, *Egr-1* transcription is increased by oxidative stress and ionizing radiation due to six serum responsive elements, the three most distal of which are functional in the response to X-rays[21]. The rapid direct induction of *Egr-1* by ionizing radiation suggests that this response is part of the primary response of cells to radiation and not a secondary phenomenon as a result of radiation-induced alterations in the cell cycle.

S phase

Ionizing irradiation of mammalian cells slows down the progression through S-phase, due to a depression in the rate of initiation of replicon clusters. This response is biphasic with a steep (radiosensitive) component at lower doses resulting from inhibition of replicon initiation[100,119,169], and a shallow (radioresistant) component at higher doses, due to the inhibition of DNA chain elongation[173]. Estimation of target size led to the hypothesis that a single radiation hit blocks the initiation of not just one but an entire cluster of replicons[119].

H-*ras*- and v-*myc* transformed rat embryo fibroblasts gain significant radioresistance and show much greater inhibition of replicon initiation after X-irradiation compared to v-*myc*-transformed cells; no significant difference could be observed in the inhibition of chain elongation[170]. These experiments indicate a role for the *ras* proto-oncogene in the signal transduction pathway which controls the inhibition of DNA synthesis. The inhibition of replicon initiation by staurosporine, an inhibitor of the divergent members of the protein kinase family[171,46], confirms the action of the *ras* oncogene in the signal transduction pathway.

The methotrexate-resistant Chinese hamster ovary (CHO) cell line, CHOC 400, has a highly amplified dehydrofolate reductase (DHFR) domain and does not display the late G1 arrest after irradiation with 9 Gy. However, within 30 min of irradiation, the initiation of the DHFR locus is completely inhibited and does not resume for 3–4 hours[84]. The lack of G1 arrest but successful inhibition of DNA replication implies a p53-independent, S-phase damage-sensitive pathway most likely including the activation of $p34^{cdc2}$ kinase as a key element.

The influence of ionizing radiation on some other factors regulating the replication system is not established. The chromatin-associated product of the gene *rcc1* (regulator of chromosome condensation) forms a complex with the small *ras*-related nuclear protein Ran/TC4 in HeLa cells, and besides its function in the regulation of the initiation of DNA replication it is also involved in the control of mitotic events. The loss of the RCC1 gene product leads to the inappropriate activation of $p34^{cdc2}$, premature chromosome condensation, and spindle formation independent of the completion of DNA replication[6]. The 125 kDa protein BM28 is also considered to be involved in DNA replication. Antibodies raised against recombinant BM28 microinjected during G1 phase inhibit DNA synthesis, while injections in S phase have no effect[166].

Both the initiation and elongation stages of DNA replication depend on the replication protein A (RPA) as shown in *in vitro* SV40 replication[9,36,178]. RPA is a protein complex composed of a p70 subunit which codes exclusively for the single strand DNA-binding activity, and subunits p34 and p11 of unknown function[9,35]. Under normal conditions the p34 subunit of the RPA is phosphorylated late in G1-S transition and throughout S and G2 phase of the cell cycle[26,30]. Exposure of human cells to a high dose of ionizing radiation induces phosphorylation in G1 which is mediated by a *cdc2*-type protein kinase[93].

In addition to the inactivation of cyclin-dependent kinase complexes during G1-S transition, $p21^{WAF1/CIP1}$ directly blocks DNA replication by inhibiting PCNA, a key factor in the replication machinery[128,186]. Excess PCNA protein removes this inhibition[90]. However, PCNA is also required for DNA synthesis during nucleotide excision repair of damaged DNA[145]. It would seem that the PCNA-dependent excision repair complex with the DNA polymerases δ und ε is resistant to the inhibition of $p21^{WAF1/CIP1}$, whereas the process of long primer extension synthesis during DNA replication, when PCNA together with replication factor C facilitates the loading and processing of DNA polymerase δ, is sensitive to the inhibition of $p21^{WAF1/CIP1}$.

G2 and the G2-M checkpoint

The exposure of mammalian cells to ionizing radiation is followed by a division delay (ref. 147; for review see refs 99, 164). After exposure to 5 Gy, HeLa S3 cells delay cell division for 8 hours due to a decrease in the rate of DNA synthesis, while the rate of entry into S remains unchanged[116,117]. Continuous labelling of HeLa S3 cells with ^{3}H-thymidine following irradiation and determination of the percentage of labelled metaphases, revealed a G2 delay proportional to the dose[182]. However, each phase of the cell cycle was not equally sensitive to radiation-induced division delay. Irradiation of HeLa S3 cells with 3 Gy in S or G2 showed maximal G2 delay, whereas irradiation in early S caused only a slightly prolonged S phase. No significant change in the G1 phase was observed under any conditions[161].

Mammalian cells labelled with the thymidine analogue 5-bromodeoxyuridine (BUdR) showed enhanced X-ray-induced mitotic inhibition[140]. In synchronized and BUdR-labelled human kidney T-cells, an X-ray-induced mitotic delay was due to a small increment in the G2 delay when compared with normal cells (in contrast to a greatly increased mitotic delay in BUdR cells after exposure to UV radiation, due to a lengthening of the S-phase). Thus the mechanism responsible for the mitotic delay following X-irradiation seems to be primarily concerned with the completion of the cell cycle and less with the repair of DNA damage[139] in contrast to the more unique DNA damage signal generated by UV damage. The signal generated by X-ray damage would appear to interfere less with progression through the cell cycle. An explanation could be the structural similarities of ss and ds breaks with DNA structures found during DNA replication.

The G2-M checkpoint was initially described as a G2 delay following DNA damage caused by ionizing radia-

tion in mammalian tissue cultures[168]. This G2 delay could be eliminated by the addition of caffeine to the culture medium which increased radiosensitivity and the accumulation of cytotoxic chromosome aberrations in cells[121].

Few factors have been identified that are directly involved in the regulation of the G2-M checkpoint. The transition from G2 to M is not blocked in cells with p53 mutations or in a subset of Bloom's Syndrome cells which show no induction of p53 after exposure to X-ray[71,79]. p53as, a variant protein of p53, generated by alternatively spliced p53 mRNA has so far been identified only in rodent cells. p53as has 17 altered amino acids at the C terminus compared with the wildtype, which provides p53as with efficient DNA-binding ability without activation. p53as is preferentially associated with the G2 phase of the cell cycle and comprises up to 30% of total p53 transcripts in normal cells[179]. It seems that in rodents p53 and p53as together carry out the fuctions of the *p53* gene.

The role of the retinoblastoma (*Rb*) gene product as part of the regulation of the G2-M checkpoint is more secured[113]. Overproduction of the Rb protein, after the G1-S boundary, causes accumulation of cells in G2. This accumulation of cells in G2 is accompanied by an augmentation of phosphorylated Rb protein, catalyzed by $p34^{cdc2}$ and other kinases[70,87,158]. Cell cycle arrest is also accomplished by Rb by its binding to several proteins involved in cell cycle progression, like BRG1[28].

Ionizing radiation induces two different modes of cell death: necrosis and apoptosis. Necrosis occurs several cell divisions after irradiation and is characterized by increased plasma membrane permeability, decline in protein synthesis, swelling and autolysis. In contrast, programmed cell death, or apoptosis, originally described in interphase lymphocytes, is characterized by a condensation of the cytoplasm, increased membrane permeability, and nonrandom degradation of nuclear DNA into oligonucleosome sized fragments[31,180]. The apoptotic process can be activated in certain cell types by p53 and this activity is inhibited by the activity of two oncoproteins, adenovirus E1B and Bcl-2. Interestingly, both oncoproteins block the transcriptional repression of p53, which raises the possibility that the apoptotic process might be induced by a repression of the transcription of certain genes[142]. Irradiation of murine hybridoma cells triggers the start of apoptosis after G2 arrest; apoptosis is fully expressed in the subsequent G1 phase and can be enhanced by substitution of nuclear DNA with bromodeoxyuridine[172]. CHO cells, arrested in G2 by cis-diamminedichloroplatinum (II), undergo aberrant mitosis with subsequent apoptosis. The addition of caffeine to G2-arrested cells induces dephosphorylation of $p34^{cdc2}$ and dramatically accelerates the events leading to apoptosis, which cannot be inhibited by a block of the protein synthesis in G2 with cycloheximide[23]. These results demonstrate that DNA damage stimulates a long-lived signal that controls the expression of apoptosis even in subsequent phases of the cell cycle. The involvement of $p34^{cdc2}$ phosphorylation indicates that key proteins that are required for the apoptotic process are also required for normal cell cycle progression.

How does the regulation of the cell cycle differ in ataxia cells?

The understanding of the basic mechanisms of the cell cycle has been helped immensely by the investigation of yeast mutants and the cloning of the responsible genes. However, the extrapolation of yeast data to mammalian systems is difficult due to the different control mechanisms which have evolved in mammalian systems, unparalleled in single cell organisms.

Mammalian systems have now been developed in which single gene mutations alter the cellular response to ionizing radiation. At least nine complementation groups for radiation-sensitive Chinese hamster cells[67,162,185], scid mice[42,78] and possibly 15 complementation groups in human radiation-sensitive cells including cells from patients suffering from ataxia telangiectasia, Nijmegen breakage syndrome[66], Alzheimer's disease and Down's Syndrome[15,105] have been identified. The cloning and/or characterization of the responsible gene(s) will help to determine the genetic basis of these primary neuronal degenerative disorders[165,69,156,138].

The most comprehensive data on the cell cycle alterations after cytotoxic stress in human X-ray-sensitive cell lines come from cells from patients suffering from ataxia telangiectasia (AT). AT is an autosomal recessive disease characterized by hypersensitivity to ionizing radiation, predisposition to cancer, chromosome instability and immune dysfunction (for review see refs 44, 51, 11). Four complementation groups have been identified in AT families[66]. At present there are stronger indications for genetic homogeneity of the AT locus on chromosome 11q23-23[101,45,132], than for separate gene loci in two or perhaps three AT complementation groups[135]. Cells from AT patients are extremely sensitive to killing by ionizing radiation[159]. Once exposed to irradiation, in contrast to normal cells, AT cells do not activate transcription of c-*myc* or *XRCC1*[146]. However, in AT fibroblasts there is a 14-fold overinduction of tissue-type plasminogen activator following irradiation[41]. In normal cells ionizing radiation induces a specific DNA-binding protein of 70 kDa, undetectable in unperturbed cells but constitutively expressed in AT cells[148,160], as if the cells were under permanent stress. A consistent feature of cultured AT fibroblasts is the continuation of DNA synthesis after irradiation, owing to a failure to downregulate replicon initiation[121,73,65,104]. The inhibition of replicon initiation appears to be an active process which requires a gene product defective in AT.

All attempts to identify a clear cut DNA repair defect in AT cells have been thwarted. After low dose irradiation of stimulated blood lymphocytes from AT patients, an increased level of initial chromosome damage compared to normal cells was detected, with AT heterozygote cells exhibiting intermediate levels[123,16].

The religation of dsb is functional in AT cells, but there is a reduction of the fast component of strand break repair[124,81]. Double strand breaks induced by X-ray or restriction endonucleases in AT lymphoblastoid cells are converted into chromosomal aberrations at a higher rate than in normal cells[92]. This increased tendency to convert DNA damage into chromosome damage can also be observed after treatment of AT lymphoblastoid cells with radiomimetic drugs and is particularly pronounced in G1 phase of the cell cycle[122,123].

The religation of restriction endonuclease-treated plasmids, either stably transfected into AT cells, or passed through AT cells as shuttle vectors, or incubated with nuclear extracts, proved to be less efficient and the plasmids suffer about twice as many mutations compared to normal cells; in AT cells, this process of mis-rejoining results in a preference for deletions at the site of the dsb, and the mis-rejoining factor was shown to be semi-dominant[43,127,134,157]

These mis-rejoining events involved deletions between short direct repeats, pointing to a defect in a non-conservative recombination mechanism[112,163]. This conclusion is supported by the fact that plasmids stably integrated in AT cells show a 30–200-fold increase in spontaneous intrachromosomal recombination[102]. However, there is no abnormality in V(D)J recombinase activity in AT cells (ref. 60; for review see ref. 76).

Cell cycle progression of AT cells following exposure to ionizing radiation

The hypersensitivity of AT cells to ionizing radiation has been linked to the inability of these cells to arrest in G1-S, S or G2-M phase of the cell cycle[184,121,105] (for review see ref. 164) and a failure to stop DNA synthesis as a response to radiation[121]. The persistence of DNA damage and/or the absence of a DNA damage signal could be the cause of a partial deregulation of cell cycle checkpoints in AT cells. However, the suggestion that the radiosensitivity of AT cells derives from a failure of the cells to initiate cell cycle delay[121] has been weakened by two findings: i) the delay of cell cycle progression in AT cells does not enhance survival after exposure to ionizing radiation[20], and ii) chromosome damage can be detected in AT cells immediately after exposure to radiation without progression through the cell cycle[19].

As a result of DNA-mediated gene transfer into AT cell lines, followed by X-ray selection, radioresistant 'AT' cell lines have been obtained[88,69,77,83,95,17,5,102]. Most of these gene transfer experiments were based on the AT5BIVA cell line[22] and the isolated radioresistant AT derivatives were co-isogenic to various degrees with the parental cell line. Parental AT5BIVA and radioresistant 'AT' cells thus established a system of cell cycle mutants, the comparison of which will help to reveal the central paradigms of cell cycle regulation after DNA damage in mammalian systems.

Alteration in G1 and S phase of AT cells after exposure to radiation

Ionizing radiation induces a dose-dependent G1 arrest in normal radioresistant cells[91] which is not found, or is expressed to a lesser extent, in homozygous AT cells[110,137,4]. AT heterozygous cells overaccentuate the G1 arrest as do Retinoblastoma mutant cell lines[109]. The induction of the gene products GADD45, MDM2 and $p21^{WAF1/CIP1}$, whose transcriptional activation is dependent on p53[72,14,33], is delayed in AT cells compared with normal cells[12]. This suboptimal induction is also found at the transcriptional level of *gadd45* and *mdm2*[125,129]. The possibility that p53 impairment could be the cause of the radiosensitivity of AT cells[72] has been eroded by the finding that AT cells are not deficient in p53, its induction is just delayed following γ-irradiation as well as after treatment with inhibitors of cell cycle progression such as mimosine and aphidicolin[97,74,111]. The abnormally slow induction of the p53 DNA-binding activity in electrophoretic mobility shift assays also confirms the suboptimal regulation of p53 in AT cells[129]. It is important to stress that this delay of p53 response in AT cells cannot be dismissed as an insignificant difference. The combination of the AT cells' failure to inhibit DNA replication and to induce the stress response system efficiently after exposure to ionizing radiation would contribute to genetic instability and decreased survival rate.

In addition to the suboptimal regulation of p53 after γ-irradiation, the phosphorylation of the Replication protein A (RPA) p34 subunit during the G1 phase of the cell cycle is delayed in AT cells compared to normal cells[93]. This phosphorylation is p53-independent and requires the binding of RPA to single stranded DNA. The slow progress of p34 phosphorylation in AT cells could therefore be the result of a lesser accessibility of RPA to ss DNA in AT cells compared with normal controls. SV40 transformation of human skin fibroblasts from normal as well as from AT donors led to an overexpression of p53, c-*myc*, Ki-*ras* and c-*raf*, which coincided with a significant increase in radioresistance[98]. However, the binding of T-antigen to p53 results in the abolition of p53 transcription factor activity[97], which argues against an increased p53-dependent transcription of stress response genes.

The activation of programmed cell death as a response to DNA damage is mediated via p53 protein and the inhibition of this pathway increased the cellular radioresistance[96,18]. AT cells, including SV40-transformed cell lines, have been shown to apoptose following exposure to ionizing radiation although apoptosis is delayed compared to normal cells (H.L. unpublished results; ref. 102). Thus it seems unlikely, that the increased radioresistance of normal and AT fibroblasts after SV40 transformation is due to the inactivation of the apoptotic pathway.

Alterations in the G2 phase of AT cells after exposure to ionizing radiation

Previous findings concerned with the alterations to the G2 phase of AT cells after exposure to ionizing radiation remain controversial. In one series of experiments, normal cells exposed to a low dose of X-rays showed a transient G2-M arrest followed by an inhibition of entry into S-phase, but AT cells were resistant to these arrests[133]. Other investigators found an increased G2 block in AT after exposure to X-rays or fast neutrons[62,151,2,95,59], which was less pronounced in AT heterozygotes[85]. It seems that irradiated AT cells exit from the G2-M block more slowly than normal cells. Factors controlling the exit from G2-M block could be defective in AT cells and the prolonged accumulation of AT cells in G2-M might not be related to radiosensitivity[58]. This discrepancy of AT cells arresting or not arresting in G2 after irradiation, could have arisen because of differences in the cell cycle phase the cells were in at the time of irradiation: irradiation of AT lymphoblastoid cells in G1 or S results in an increased G2 delay compared to normal cells; irradiation of AT cells in G2 reduces the G2 phase and the following S phase compared to wild-type cells; the accumulation of AT fibroblasts in G2-M is due to the fact that only 2.7% of the AT cells pass through the block 24 hours after irradiation compared to 23–28% of normal cells[164,4,3,141].

Perhaps the strongest indication of the importance of the G2 block for the AT phenotype is given by the fact that repair proficient and radioresistant derivatives of AT cells[83,95] overcome the G2 block after exposure to 4 Gy in contrast to their radiosensitive parental AT5BIVA cells (see table 1). The radioresistant 'AT' cells represent different ways of overcoming radiosensitivity. These different solutions have a molecular basis that might depend on the genetic background of AT cells (like the cosmids complementing AT-D radiosensitivity isolated by Kapp and Painter; ref. 69) or might reflect a general mechanism, which would also be operating in normal cells. For the moment these cell lines represent a spectrum of cell cycle mutants, which respond differentially to cytotoxic stress. An investigation into the molecular processes underlying these responses will make it possible to identify the chain of interacting cellular factors, ranging from the initial signal of radiation damage to its repair and the optimization of cellular survival. At present, the correlation between cell survival and cell cycle delays remains elusive.

Conclusions

Alterations to the cell cycle of mammalian cells after exposure to ionizing radiation are major contributors to increased cellular survival. These alterations are determined by cell cycle checkpoints, regulating the transition from G1 to S and G2 to M phase, and integrating the behaviour of the DNA replication complex with signals of cytotoxic damage. The G1-S checkpoint is

Table 1. Cell cycle characteristics in cell lines complementing the AT-D defect with respect to radioresistance. Following gene transfer into AT5BIVA cells and X-ray selection, a series of radioresistant cell lines was isolated, which differ in their DNA synthesis and cell cycle regulation after exposure to ionizing radiation.

Reference	Complementation achieved by	Radioresistant DNA synthesis	Cell cycle alterations
Lehmann et al.[88]	naked DNA-mediated gene transfer	intermediate, close to AT phenotype	nd*
Kapp and Painter[69]	cosmid-mediated gene transfer	intermediate, close to AT phenotype	nd*
Komatsu et al.[77]	chromosome-mediated gene transfer	corrected to wild-type level*	nd*
Lambert et al.[83]	chromosome-mediated gene transfer	corrected to wild type level	no G2 block after 4 Gy, G2 delay identical to wild type
Lohrer et al.[95]	cell-mediated gene transfer	intermediate, close to wild type level	no G2 block after 4 Gy, G2 delay identical to wild type

nd = not done, * personal communication.

critically dependent on p53 but also on $p21^{WAF1/CIP1}$, GADD45 and MDM2. p53 protein also seems to be involved in the regulation of the G2-M checkpoint together with hyperphosphorylated Rb protein catalyzed by $p34^{cdc2}$ and other kinases. The initiation of DNA replication is dependent on the interactions of RPA, PCNA and DNA polymerase δ. Of these factors, RPA, p53, GADD45 and cyclin B-CDK-1 also respond to DNA damage caused by ionizing radiation. Ataxia telangiectasia cells are impaired in the response of p53 and p53-dependent factors to ionizing radiation. However, the suboptimal induction of p53 is not responsible for the abnormalities of the AT phenotype. The combination of X-ray sensitivity and the continuation of replicon initiation after exposure to ionizing radiation in AT indicate that the signal chain from DNA damage to the cell cycle is the most important parameter for cell survival. This signal chain malfunctions in AT cells. Comparisons of the regulation of the cell cycle between AT cells and their radioresistant derivatives after irradiation will make it possible to identify the factors governing the cellular response to ionizing radiation.

Epilogue

After the submission of this manuscript, the identification of the gene responsible for AT by positional cloning was reported[138]. The *ATM* gene has a coding region of 12 kb and more than 40 different mutations have been identified. Computer analysis of the protein sequence revealed similarities to i) phosphatidylinositol-3 kinases (PI-3 kinases), regulators of cellular growth control and cell signalling, ii) yeast Rad3, involved in the G2-M cell cycle checkpoint after DNA damage, iii) yeast ESR1, required for DNA repair and meiotic recombination, and iv) the yeast TOR1 and TOR2, a family of signal transducers involved in the control of the G1 phase of the cell cycle. Earlier, complementation analysis, based on the fusion of different AT cells followed by the analysis of the radiation-resistant DNA synthesis, led to the assumption of four complementation groups, i.e. four genes or four functional modules in one big gene product[66]. However, the determination of the mRNA sequence of the ATM gene revealed no evidence of more than one AT gene: all AT cases investigated had a mutation in the ATM gene and cells from the complementation groups C and E showed the identical mutation. Thus, at the moment it seems impossible to reconcile the molecular data with the classical complementation experiments. At the cellular level the correction of the AT phenotype by transfer of the *ATM* gene has so far not been possible due to the uncloned 5'-end of the *ATM* gene. The putative biochemical functions in DNA repair and the cell cycle ascribed to the *ATM* gene product, and its interactions with other cellular factors outlined in this review, remain to be investigated.

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