

## Short reports

# Cell cycle dependent DNA break increase in ataxia telangiectasia lymphoblasts after radiation exposure

B Humar, H Müller, R J Scott

### Abstract

**The most striking feature of ataxia telangiectasia (AT) cells is their profound sensitivity to ionising radiation. A deficiency in the rejoining of radiation induced DNA breaks has been suggested to be responsible for AT radiosensitivity; however, the existing literature is controversial. A subpopulation, which is present in irradiated AT lymphoblasts, but rarely in controls, has been reported previously. The cells that make up this subpopulation harbour highly fragmented DNA and are responsible for the overall increase in DNA breaks soon after irradiation in AT lymphoblasts. This study examines the influence of the cell cycle on the highly damaged subpopulation. The frequency of highly damaged cells was highest when AT lymphoblasts were irradiated during the G2/M phase. In contrast, AT lymphoblasts irradiated during the G0/G1 phase displayed a frequency similar to control cells. Thus, only G2/M and to some extent S phase cells contribute to an increased DNA break number in AT lymphoblasts early after irradiation. These findings might explain several inconsistencies reported in the literature.**

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Ataxia telangiectasia (AT) is classified as an autosomal, recessively inherited, chromosome breakage syndrome. The main symptoms of AT include neurological, developmental, and immunological abnormalities in addition to a high risk of cancer. The disease is characterised by extreme clinical and cellular radiosensitivity.<sup>1</sup> The protein encoded by the gene mutated in AT (ATM) belongs to a family of large proteins known to be crucial regulators of cellular responses to DNA damage.<sup>2,3</sup> Although a variety of defects such as impaired cell cycle checkpoints have been reported for irradiated AT cells (reviewed by Jorgensen and Shiloh<sup>2</sup> and Lavin and Shiloh<sup>3</sup>), the reason for their radiation susceptibility remains unclear. To determine whether impaired repair of radiation

induced DNA damage contributes to radiosensitivity, several studies have investigated the capacity of AT cells to rejoin broken DNA after irradiation. However, no clear repair deficiency could be established in AT—some groups have observed a normal rejoining capacity,<sup>4–9</sup> whereas others have detected increased DNA break numbers in irradiated AT cells compared with controls<sup>10–13</sup> at early times after irradiation.

We have previously analysed the radiation response of AT lymphoblastoid cells.<sup>14</sup> Using the comet assay, we found an increased number of DNA breaks in AT cell populations soon after irradiation. However, the measurement of DNA breaks in individual cells revealed the presence of a subpopulation of highly damaged AT lymphoblasts, which are responsible for the increase in the overall number of DNA breaks compared with irradiated control populations. The cells that make up this subpopulation (HDCs; highly damaged comets) are characterised by a very high degree of DNA fragmentation, which is at least in part caused by early, inappropriate apoptosis of AT cells. Apart from this subpopulation, AT lymphoblasts rejoin DNA breaks with normal capacity. Thus, the existence of a highly damaged subpopulation helps to explain seemingly contradictory notions of normal repair,<sup>4–9</sup> but increased DNA break numbers soon after irradiation.<sup>10–13</sup>

However, another reason for discordant observations with respect to DNA repair in AT might be the use of cycling versus non-cycling AT cells in different reports. Our study was carried out to determine whether the cell cycle influences the numbers of DNA breaks detected in irradiated AT cells. The frequency of HDCs in cell cultures irradiated during different cell cycle phases was measured. We show that cell cycle position is an important parameter affecting DNA break numbers in irradiated AT lymphoblasts.

### Materials and methods

#### CELL LINES AND CULTURE

Epstein-Barr virus (EBV) transformed human B cells (GMO9582, GMO8436; AT homozygous) were obtained from the Human Genetic Mutant Cell Repository, Camden New Jersey, USA. The control cell line WIMA-1 was derived by Ficoll gradient separation and EBV transformation of heparin

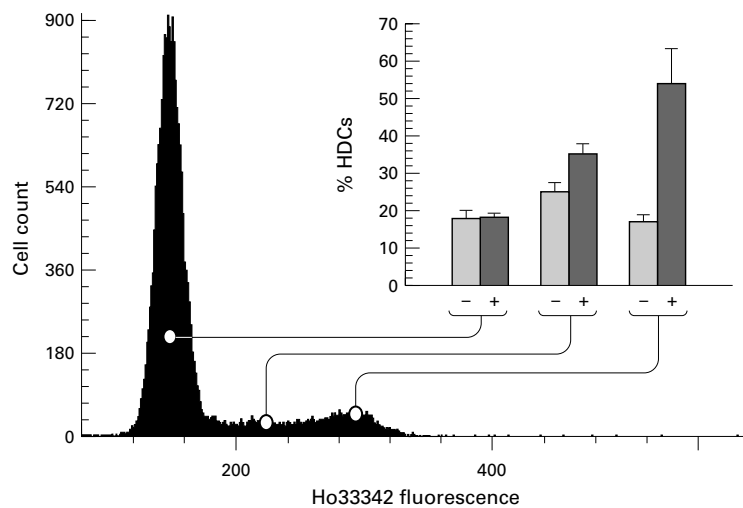
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**Figure 1** Cell cycle distribution and corresponding highly damaged comet (HDC) frequencies in ataxia telangiectasia (AT) cells. AT lymphoblasts (GMO9582) were sorted according to their cell cycle phases, exposed to 6 Gy, incubated for two hours, and comet analysis was performed. Their HDC frequencies are shown in the integrated chart. Minus and plus signs indicate unirradiated and irradiated samples, respectively. Error bars refer to the SEM of four independent observations. The radiation induced HDC frequencies (corrected for the corresponding unirradiated samples) of the individual cell cycle phases differed significantly ( $p < 0.025$ ; Mann-Whitney U test).

treated blood samples from a healthy human donor (age > 20 years, clinically diagnosed as unaffected, asymptomatic, no family history). Cells were maintained as described previously.<sup>14</sup>

#### IRRADIATION

Cells embedded in agarose were irradiated in ice cold phosphate buffered saline with  $\gamma$  rays at a dose rate of 0.5 Gy/minute using a <sup>60</sup>Co source as described previously.<sup>15</sup>

#### ALKALINE COMET ASSAY

The alkaline comet assay was used to detect single and double stranded DNA breaks in individual cells and was performed as described previously.<sup>14</sup>

#### ANALYSIS OF SLIDES

Slides were analysed using a Zeiss fluorescence microscope attached to a CCD camera (Nu 200; Photometrics, Huntington Beach, California, USA). The comet head was separated from the tail with a circle and the fluorescence intensity of the comet was measured using IPLab Spectrum software (Signal Analytics, Stockholm, Sweden). HDCs were defined as comets with: (1) a typically shaped tail, and (2) containing > 90% of the comet's DNA. The percentage of HDCs was determined by viewing 500 to 1000 comets/sample.

#### CELL CYCLE ANALYSIS

Twenty million cells were incubated at 37°C for 30 minutes with 5  $\mu$ g/ml Hoechst33342 dye, spun down, and resuspended in ice cold RPMI complete medium (at a concentration of  $2 \times 10^6$  cells/ml), supplemented with 5  $\mu$ g/ml Hoechst33342 and 5  $\mu$ g/ml propidium iodide. Analysis was performed using a dual laser FACS Vantage (Beckton Dickinson, Franklin Lakes, New Jersey, USA) equipped with 488 and 360 nm lasers for excitation, in addition to

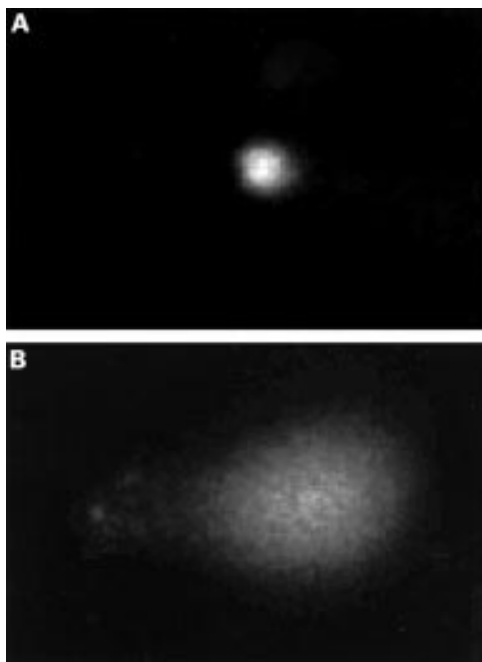
585/42 and 424/44 nm filters for the detection of PI and Hoechst33342 fluorescence, respectively. According to the Hoechst33342 fluorescence histogram, living cells were sorted at 4°C into their corresponding cell cycle positions. Cells were immobilised in agarose on culture slides, irradiated with 6 Gy, incubated for two hours at 37°C, and then used in the comet assay.

#### Results and discussion

The aim of our study was to examine factors that could influence the number of DNA breaks in irradiated AT cells and thereby contribute to a more complete understanding of the AT radiation response.

To unravel a possible relation between the HDC subpopulation and the cell cycle, AT lymphoblasts (GMO9582) were sorted according to their cell cycle position, irradiated, and analysed with the comet assay two hours later. At this time point, the difference in HDC frequencies between AT and control cells is most pronounced.<sup>14</sup> As shown in fig 1, AT lymphoblasts irradiated during the G2/M phase of the cell cycle were most susceptible to the induction of HDCs. Irradiated S phase cells also displayed modest increases in HDC frequency. In contrast, the HDC frequency of G0/G1 cells was only slightly increased after irradiation. Similar HDC frequencies in the different cell cycle phases were obtained for another AT cell line (GMO8436: G0/G1, 2% over a background of 19%; S, 7% over 26%; G2/M, 35% over 21%; two independent observations). In contrast to AT lymphoblasts, no dependence of HDC frequencies upon cell cycle position could be seen for a control cell line (WIMA-1), and frequencies were in the range of 0–4% above background (range, 18–22%) for all phases (two independent observations). Because three other control cell lines (LUMA-1, GMO3657, and HL-60) have radiation induced HDC frequencies in the same range as WIMA-1, regardless of the cell cycle position,<sup>14</sup> we did not sort additional controls. Figure 2 shows examples of an HDC and a comet with intact DNA.

The reason for cell cycle dependency is not clear. It is probable that radiation induced DNA damage provides a trigger for the induction of HDCs. The ploidy content in G2/M may thus result in a greater probability of generating DNA breaks, leading to an increased HDC frequency. However, the HDC frequency is only slightly raised in irradiated G0/G1 cells, whereas it is in the range of 40% for G2/M cells. Therefore, additional factors are probably involved. In AT cells, DNA breaks appear to be converted into chromosome breaks with a much higher efficiency than in controls.<sup>15</sup> The condensation of chromatin during late S and G2 might result in an excess of chromosomal breaks, thereby increasing the HDC frequency specifically in these cell cycle phases. Sensitivity to DNA breaks in general might also be one explanation for the increased background HDC frequency in unirradiated samples of S phase cells (fig 1), because basal



**Figure 2** Microscopic appearance of highly damaged comets (HDCs). (A) A comet with very low amounts of DNA damage, showing almost no comet tail. (B) A comet typical of the highly damaged subpopulation; the comet head (to the right) is hardly visible, with almost all of the DNA migrated into the comet tail. Both comets were scored in ataxia telangiectasia samples two hours after irradiation.

DNA break values will be increased in this phase as a result of the presence of replication forks.

Furthermore, AT lymphoblasts are known to have impaired G2/M checkpoint regulation after irradiation,<sup>16</sup> which might contribute to the high HDC frequency in this cell cycle phase. The appearance of HDCs has been shown to correlate with increased p53 independent apoptosis in irradiated AT lymphoblasts.<sup>14</sup> Intriguingly, radiation induced, p53 independent apoptosis is often associated with the G2/M cell cycle phase.<sup>17–19</sup> In addition, caffeine treatment of cells lacking p53 radiosensitises cells via increased apoptosis owing to G2/M arrest abrogation.<sup>20</sup> Accordingly, AT lymphoblasts have defective p53 function,<sup>2,3</sup> and caffeine has been suggested to mimic the AT phenotype with respect to G2/M checkpoint and radiation sensitivity in lymphoblasts.<sup>21</sup>

Although not clarifying AT radiosensitivity as such, the data presented in our study may help to understand discordant reports about DNA break repair in AT.

Our results indicate that an increase in HDCs and thus in DNA breaks at early time points (< 4 hours) after irradiation will occur in cycling AT cells (at S and G2/M phase), but not in resting AT cells (at G0/G1). Concordantly, an increase in DNA breaks early after radiation exposure was noted by others exclusively in cycling AT cell cultures,<sup>10–13</sup> and never in AT cells held in G0/G1.<sup>4,5,14</sup> Therefore, the appearance of HDCs early after irradiation in cycling cells only can explain the seeming discrepancy between existing reports on the early radiation response of AT cells. Because we have

previously shown that the DNA break increase within four hours after irradiation is not the result of a repair deficiency, but appears to be related to an increased frequency of radiation induced apoptosis, induction of cell death might be an important parameter in the abnormal radiation response of cycling AT cells. Normal DNA metabolism and ordinary environmental insult will produce intrinsic DNA damage, which might induce an increased frequency of cell death in cycling AT cells, possibly explaining the high spontaneous cell loss in AT cell cultures.

The cellular defects that underlie the abnormal radiation response of AT cells are thought to be closely associated with other features of the AT syndrome. Intriguingly, some of the clinical symptoms of AT<sup>1</sup> might be related to inappropriate induction of apoptosis. Raised  $\alpha$  fetoprotein is a consistent finding in patients with AT and might be indicative of liver damage, as sometimes manifested by liver cirrhosis. Other degenerative changes can occasionally be seen in gonadal, adrenal, and lymphoid tissues (lymphocytopenia), which all share a high cellular turnover rate and thus might be susceptible to increased loss of cycling cells. Endogenous DNA damage is increased in AT cells and might accelerate the induction of apoptosis by intrinsic DNA breaks. One important function of ATM is thought to be the detection of DNA breaks,<sup>22</sup> and the loss of ATM might result in inappropriate removal of cells without discrimination of damaged from undamaged ones. In this way, low numbers of intrinsic DNA breaks may provide a persistent trigger for the gradual loss of cycling cells, sometimes resulting in degenerative tissue changes. The cause of ataxia, the leading symptom of AT, is not known; however, cerebella abnormalities can be seen in the brains of patients with AT at postmortem examination and include an apparent loss of Purkinje cells.<sup>1</sup> Purkinje cells are resting; however, in mice, the *Atm* gene is highly expressed during embryonal neurogenesis in areas undergoing mitosis and in areas containing Purkinje cell precursors, but hardly at all in terminally differentiated postnatal Purkinje cells.<sup>23</sup> If the expression pattern of ATM is similar in the human brain, it is possible that loss of ATM affects cycling neurone precursors primarily. Defects associated with cycling AT cells might therefore contribute to the paucity of Purkinje cells seen in patients with AT. Others have proposed that neurodegeneration in AT might be the result of an immunological imbalance as a result of accelerated cell death, and might be overcome by the addition of appropriate serum survival factors.<sup>24</sup> Intriguingly, high serum concentrations in AT cell cultures can protect against the induction of HDCs,<sup>15</sup> suggesting that survival factors might be of some therapeutic benefit. These factors should be identified and carefully evaluated for their specificity, because the price of reduced degeneration might be an increased likelihood of malignancies in patients with AT.

Taken together, we have shown that the HDC subpopulation is only apparent when AT

lymphoblasts are irradiated during the G2/M or S phase of the cell cycle, but not during the G0/G1 phase. Thus, the HDC phenotype appears to be associated with cycling AT cells, explaining the increase in DNA break numbers observed early after irradiation in cycling AT cells only. These findings help to integrate previously conflicting observations that exist in the literature into a common framework.

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