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Overexpression of Rb and E2F-1 in Ataxia-Telangiectasia Lymphocytes*

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AT cells exhibit defective cell cycle regulation following DNA damage. Previous studies have shown that induction of p53 and p21 proteins are delayed in response to ionizing radiation, resulting in the failure of G1/S checkpoint in AT cells. In this study, further investigation of the molecular mechanisms underlying G1/S phase progression in AT cells was conducted. Exponentially growing normal and AT cells were exposed to 2 Gy of ionizing radiation and the expression levels and functional activities of Rb and E2F-1 proteins were determined. We observed overexpression of hyperphosphorylated Rb and E2F-1 proteins in AT cells, which was unaffected post-irradiation. Furthermore, gel shift assays showed that E2F-1-DNA binding was constitutive in AT cells, whereas it was inhibited in control cells following exposure to ionizing radiation. The data suggests that abnormalities in the function of Rb and E2F-1 proteins may also be responsible for the failure of AT cells to arrest in the G1/S checkpoint in response to DNA damage.

Key words : Rb, E2F-1, Ataxia-telangiectasia cells, G1/S checkpoint, Iionizing radiation

INTRODUCTION

Ataxia-telangiectasia (AT) cells are hypersensitive to killing by DNA damaging agents (McKinnon, 1987; Taylor *et al.*, 1994). Furthermore, cell cycle check points are deficient, resulting in the failure of AT cells to undergo arrest of DNA synthesis following exposure to ionizing radiation (Beamish *et al.*, 1994). The failure of cell cycle check points has been suggested as a mechanism for the chromosomal instability and increased carcinogenic risks observed in AT patients (Meyn, 1995).

Recent cloning of the ATM (AT, mutated) gene has provided insight into possible mechanisms underlying the AT genetic defect (Savitsky *et al.*, 1995). ATM has homology to phosphoinositide -3-kinase (PI-3K) and DNA-dependent protein kinase (DNA-PK), proteins with postulated roles in signal transduction of cell cycle regulation and DNA damage responses (Savitsky *et al.*, 1995; Zakian, 1995). In normal cells, DNA damage results in the induction of p53 protein which participates in the G1/S checkpoint. However, expression of p53 and other inducible proteins (p21, Gadd 45, and mdm 2) are delayed in AT cells following exposure to ionizing radiation (Kastan *et al.*, 1992; Khanna and Lavin, 1993; Lu and Lane, 1993). In AT cells, the delayed induction of p21WAF1 is thought to be responsible for the lack of inhibition of cyclin-dependent kinases (cdk2 and 4) that are known to phosphorylate retinoblastoma (pRb) protein, thus resulting in the failure to arrest in the G1 phase of the cell cycle (Beamish *et al.*, 1996; Khanna *et al.*, 1995).

Phosphorylation of Rb is recognized as the critical molecular event that determines the progression of cells into S phase (Pardee, 1974; Pardee, 1989). The hypophosphorylated form of Rb (pRb) protein is complexed to E2F-1/DP-1 dimer in G0 and early G1 phases of the cell cycle (Chellappan *et al.*, 1991). This protein complex disassociates following hyperphosphorylation of Rb (ppRb) and the free E2F-/DP-1 complex binds DNA to transcriptionally activate S-phase specific genes allowing DNA replication.

In this study we observed that AT cells exhibit overexpression of hyperphosphorylated Rb (ppRb) and E 2F-1 proteins, the expression of which was unaltered by radiation treatment. In agreement with this, the DNA binding function of E2F-1 protein was constitutively active in AT cells irrespective of treatment with ionizing radiation.

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MATERIALS AND METHODS

Cell lines

Normal lymphoblastoid cell line GM00546 and GM 03715 and AT homozygous lymphoblastoid cell lines AT/A/GM11260 (ATL/A) and AT/C/GM13328 (ATL/C) were obtained from the Human Genetic Mutant Cell Repository, National Institute of General Medical Sciences, Camden, NJ. The cell line, L3 expressing no AT protein was a kind gift of Dr. Yossi Shiloh. The cells were grown in RPMI-1640 medium supplemented with 15% FBS.

Ionizing radiation treatment

Cells were exposed to 2 Gy of γ-rays from a cesium 137 JL Shepherd Mark I irradiator at a dose rate of 114 cGy/min. After exposure, the cells were maintained in culture until harvested at the indicated time points.

Western blotting and immunoprecipitation

Unirradiated and irradiated cells were lysed in a buffer consisting of 25 mM Tris pH 7.4, 50 mM NaCl, 0.5% sodium deoxycholate, 2% NP40, 0.2% SDS, 100 μg/mL Pefabloc SC (AEBSF) hydrochloride, 50 μg/mL aprotinin and 100 μg/mL leupeptin. Protein concentration was measured using the Bio-Rad protein assay reagent. SDS-PAGE analysis was performed on aliquots of 20 μg of total cell extract. Proteins were transblotted to PVDF membranes in Tris-glycine buffer. The membranes were incubated consecutively for one hour each with the specific primary antibodies and horseradish peroxidase labeled secondary antibodies. Immune complexes were detected using the Amersham ECL immunodetection reagent system. For immunoprecipitations, one hundred micrograms of total cell extract was incubated for 1 hour at 4°C with 5 μg of antibodies specific to cdk2 kinase or E2F-1 followed by precipitation with 20 μl of Protein A/G Plus agarose by rocking for 1 hour at 4°C. The immunoprecipitated protein complexes were denatured by boiling in SDS sample buffer, separated by SDS PAGE and subjected to western blotting as described earlier. The primary antibodies used in these experiments are as follows: Rb antibody, PMG3-245 form Pharmingen; E2F-1 antibody, KH95 from Santa Cruz; cdk2 antibody, M2 from Santa Cruz; and DP-1 antibody, K-20 from Santa Cruz.

Kinase assays

cdk2 protein was immunoprecipitated and pelletted by centrifugation as described earlier. The complex was washed thoroughly with kinase buffer consisting of 50 mM Hepes pH 7.5, 10 mM $MgCl_2$, 1 mM DTT. Kinase reactions were performed for 20 min. at 30°C in the kinase buffer containing 25 μM ATP, 1 μCi γ-^{32}P ATP and either 1 μg of histone H1 or 0.5 μg of GST-Rb as substrates. The reactions were terminated by the addition of SDS-PAGE sample buffer. The extent of phosphorylation was assessed by SDS-PAGE and autoradiography.

Electrophoretic mobility shift assays (EMSA)

Nuclear proteins were isolated by the method of Dignam *et al.*, 1993. Briefly, cells were allowed to swell on ice in lysis buffer containing 10 mM HEPES pH7.9, 10 mM KCl, 1 mM EDTA, 1 mM DTT, 1 mM PMSF, 1 μg/mL each of aprotinin, pepstatin, leupeptin and antipain. The nuclei were pelleted and resuspended in storage buffer containing 10 mM HEPES pH 7.9, 50 mM KCl, 300 mM NaCl and proteinase inhibitors. Following incubation on ice for 30 min. with periodic mixing, the nuclear fraction was obtained by centrifugation. Protein quantitation was performed using the Biorad protein assay reagent. Ten micrograms of the nuclear extract was incubated with γ-^{32}P-labeled E2F-1 consensus oligomer in the presence of poly dI-dC for 30 min. The DNA bound complex was separated by PAGE and the dried gel was exposed to Biomax MR2 film.

RESULTS

Previous studies have shown that the induction of p 53 and p21 proteins are delayed in AT cells following radiation as compared to normal cells (Kastan *et al.*, 1992; Khanna and Lavin, 1993; Lu and Lane, 1993). Induction of p21 in normal cells is associated with the inhibition of cdks that phosphorylate the Rb protein (Khanna *et al.*, 1995). To further investigate the molecular mechanisms underlying G1/S phase progression in AT cells, exponentially growing cells were exposed to 2 Gy of ionizing radiation and the functional activities of Rb and E2F-1 proteins were determined.

Western blot analysis of Rb protein in cell extracts of normal and AT cells demonstrates that the basal level of expression of Rb protein was substantially higher in AT cells than in normal cells (Fig. 1). Moreover, following radiation, a gradual inhibition of Rb phosphorylation with a concomitant appearance of the faster migrating hypophosphorylated form of Rb (pRb) was observed in normal cells whereas in AT cells no such inhibition was observed. This was confirmed by analyzing cdk2 function in these cells. Immunoprecipitates of cdk2 from normal and AT cells exposed to 2 Gy of γ-rays were assessed for its ability to phosphorylate GST-Rb (specific substrate) and histone H1 (non-specific substrate). Cdk2 kinase activity was inhibited in normal cells 6 hours following irradiation but not in AT cells (Figs. 2A & B). The

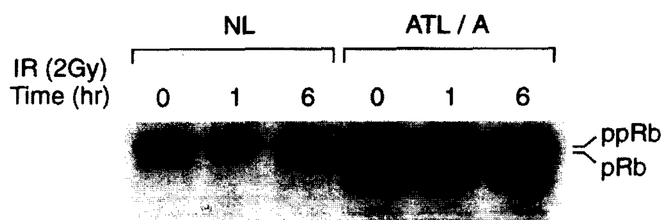

Fig. 1. Western blot analysis of Rb protein in normal (NL) and AT (ATL/A) cell at indicated times following 2 Gy of γ-rays.

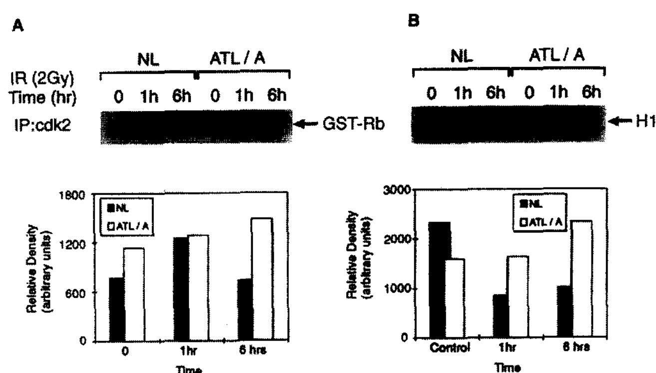

Fig. 2. Functional analysis of cdk2 in normal (NL) and AT (ATL/A) cells exposed to 2 Gy of γ-rays by *in vitro* kinase assays. Autoradiogram and densitoimetric analysis of cdk2 immunoprecipitates prepared at indicated time intervals in phosphorylating GST-Rb (A) and histone H1 (B). cdk2 protein immunoprecipitated from 100 μg of cell extract using cdk2 antibody was utilized in kinase assays in the presence of either GST-Rb or histone H1 as substrates. The reaction was separated by SDS-PAGE and subjected to autoradiography.

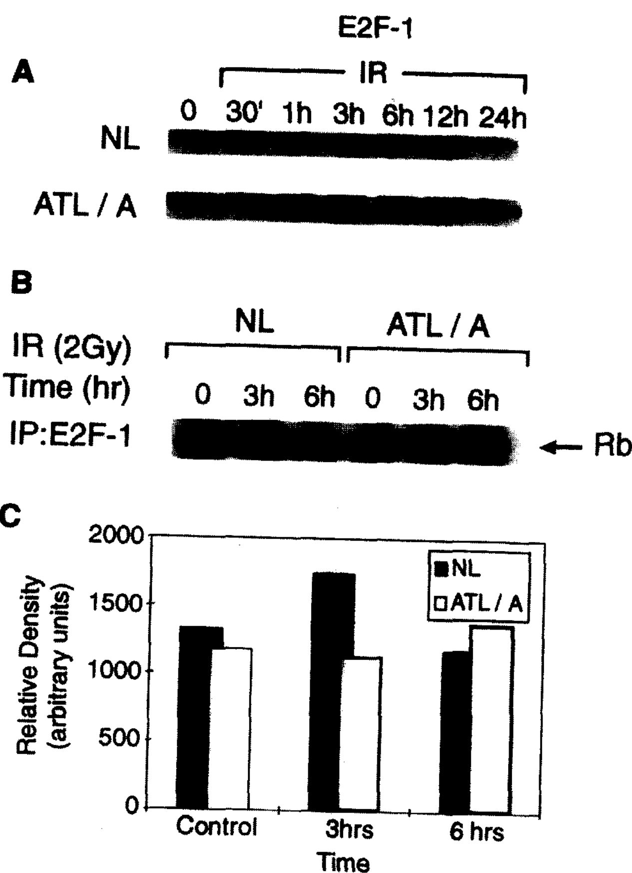

Fig. 3. Kinetics of expression of E2F-1 protein and the functional activity of Rb protein in normal (NL) and AT (ATL/A) cells irradiated with 2 Gy of γ-rays. (A) Western blotting of E2F-1 protein from cell extracts prepared at various time intervals followin radiation treatment. (B) Immunoblotting for Rb in E2F-1 immunoprecipitates prepared from cells harvested at indicated time points. (C) Densitometric analysis of the blot from Fig. 3B.

increase of cdk2 kinase activity reflects the decreased levels of inhibition mediated by lower amounts of p 21/cdk2 complexes in AT cells. This observation is consistent with previous reports (Lu and Lane, 1993; Ludlow *et al.*, 1993).

E2F-1 protein level was also high in AT cells and was not altered in response to radiation (Fig. 3A). Since the hypophosphorylated Rb protein sequesters the E2F-1 /DP-1 dimer, the functional state of pRb protein was investigated by determining the level of pRb/E2F-1 complex in these cells. As shown in Fig. 3B and 3C, an increase in the amount of pRb-E2F complex was observed in normal cells by 3 hours, whereas no significant increase was observed in AT cells.

The increased expression of both ppRb and E2F-1 proteins in AT cells suggests that the E2F-1/ DP-1 complex available for binding to promoter elements is enhanced. Electrophoretic mobility shift assay (EMSA) revealed that the basal level of E2F-1 DNA binding was significantly higher in nuclear extracts from three different AT cell lines as compared to normal cells (Fig. 4). A decrease in E2F-1 DNA binding was observed in control cells by 1 hour, but there was no inhibition in AT cells following irradiation. The specificity of the binding was confirmed by competition with cold oligomers and by the use of antibodies to E 2F-1, DP-1, or Rb for supershift. The unlabeled oligos inhibit the specific binding of the labeled E2F-1 oligomer with E2F-1 protein from unirradiated nuclear extracts of AT cells. Furthermore, E2F-1-DNA binding was markedly reduced in the presence of E2F-1 and DP-1 antibodies and to a lesser extent in the presence of Rb antibody. However, no supershift was seen with any of the antibodies which may be due to the masking of the oligo binding site on E2F-1/DP-1/pRb complex by the antibodies.

DISCUSSION

In normal cells, p53 and p21 are activated by DNA damage and regulate the activity of Rb protein, a critical determinant of S phase progression. Transcrip-

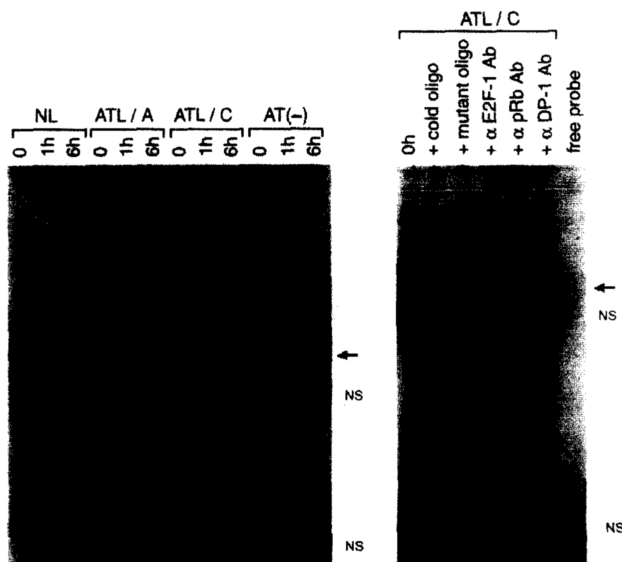

Fig. 4. Functional activity of E2F-1 protein assayed by EMs in normal (NL) and different AT cell lines (ATl/A: AT lymphoblastoid cell line, complementation group A; ATL/C: AT lymphoblastoid cell line, complementation group C; AT(-): At lymphoblastoid cell line lacking functional AT protein). Ten micrograms as nuclear extracts was incubated with γ-^{32}P labeled E2F-1 consensus binding site oligomer, separated by PAGE and subjected to autoradiography. Specificity of the binding was established by the addition of either unlabeled oligomer as a competitor or specific antibodies that would interfere with the binding in reactions using ATL/C extract.

tional upregulation of p21 by p53 results in the increased binding of p21 with cdk2 and cdk4, which are then unable to phosphorylate Rb protein. Hypophosphorylated Rb inactivates E2F-1 by forming a complex thus arresting the cells in G1 phase. Defects of G1/S checkpoint in response to DNA damage have been attributed to the defective induction of p53 and p21 proteins in AT cells (Khanna *et al.*, 1995).

In this study, we have observed overexpression of hyperphosphorylated Rb (ppRb) and E2F-1 proteins in AT cells. Furthermore, ionizing radiation treatment of these cells did not result in altered regulation of these proteins; in particular, an inhibition of Rb phosphorylation was not observed. This lack of inhibition is reflected in the kinetics of the *in vitro* phosphorylation of GST-Rb and histone H1 by cdk2 kinase from AT cells which progressively increased with time. The constitutive cdk2 kinase activity in AT cells, therefore, contributes to the continued progression of cells through G1 phase.

The functional activity of pRb protein as determined by its ability to complex with E2F-1 shows increased levels of complex in normal cells within 1 hour of irradiation. Correspondingly, E2F-1-DNA binding gradually decreased in normal cells following irradiation. However, E2F-1-DNA binding activity is markedly higher in AT cells with no inhibition following radiation. Thus, the functional activities of both Rb and E2F-1 correlates with higher level of expression of these proteins in AT cells. The higher DNA binding activity of E2F-1 in AT cells would suggest its ability to promote the transcription of E2F-1 target genes that encode proteins necessary for the entry and progression through the S phase of the cell cycle (La Thangue, 1994; Nevins, 1992).

Taken together, these data support the hypothesis that the increased level of ppRb and E2F-1 protein expression and consequently their functional activities may be partly responsible for the lack of G1 arrest displayed by AT cells. We propose that the existence of ppRb in unirradiated AT cells may be responsible for the abnormal cell cycle progression. The function of the phosphatase, PP-1, that dephosphorylates ppRb at the post-mitotic stage (Ludlow *et al.*, 1993), may be impaired in AT cells. Alternatively, because the promoter of Rb gene has been shown to contain E2F-1 recognition sequence, the overactive E2F-1 in AT cells could potentially drive the expression of Rb to high levels (Shan *et al.*, 1994).

The capacity for overexpressed E2F-1 to overcome a G1/S delay in AT cells is supported by previous findings that ectotopic expression or deregulation of E 2F-1 drives the entry of quiescent cells into S phase and promotes tumorigenesis (De Gregori *et al.*, 1995; Kowalik *et al.*, 1995; Lukas *et al.*, 1996). Of particular significance to this study is the report by De Gregori *et al.*, in which overexpression of E2F-1 in REF52 cells resulted in bypassing a G1 arrest mediated by ionizing radiation (De Gregori *et al.*, 1995). The data presented in this study support the hypothesis that abnormal regulation of Rb and E2F-1 proteins is partly responsible for the defective G1 arrest in AT cells.

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