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Abrogation of G1 Arrest after DNA Damage Is Associated with Constitutive Overexpression of *Mdm2, Cdk4,* and *Irf1* mRNAs in the BALB/c 3T3 A31 Variant 1-1 Clone

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A search of cell lines showing aberrant cell-cycle checkpoints revealed the lack of γ-irradiation-induced G1 arrest in the BALB/c 3T3 A31 variant 1-1 (A31-1-1) clone. This clone is well-known for its hypersensitivity to transformation by DNA damaging agents. p53 stabilization and *p21* mRNA induction after 8 Gy irradiation were observed, suggesting that the abrogation of G1 arrest occurred in spite of normal p53 and p21 responses by abnormal regulation of other cellular factors. Constitutive overexpression of *Mdm2* and *Cdk4* mRNAs was found, which might have contributed to the loss of G1 arrest. In addition, overexpression of a growth-suppressive transcription factor, *Irf1,* a putative key molecule in the p53-independent pathway after DNA damage, was also observed, although the relation to the loss of G1 arrest could not be elucidated.

Cell-cycle checkpoint mechanisms are known to be very important for efficient DNA-repair after DNA damage, G1 arrest being dependent on the function of wild-type p53 and p21 [1-6]. Several other factors have also been reported to affect G1 arrest induction. For example, overexpression of Mdm2 protein, a negative regulator of p53, abolishes G1 arrest [7, 8]. Phosphorylation of a tyrosine residue in Cdk4 is required for UV-induced G1 arrest [9]. Recently it was found that after DNA damage, G1 arrest is absent in interferon regulatory factor I (*Irf1*) deficient mouse fibroblast cells [10], indicating the presence of a *p53*-independent G1 arrest pathway. We also reported that poly(ADP-ribose) polymerase inhibitors suppress G1 arrest following γ-irradiation in mouse fibroblast C3D2F$_1$ 3T3-a cells [11].

For elucidating details of the cell-cycle check-point mechanisms, cell lines which show aberrant control are very useful. In the present study, we demonstrated that the BALB/c 3T3 A31-1-1 clone lacks G1 arrest following γ-irradiation despite a normal response of p53, p21 induction. Further analysis showed that this is associated with aberrant overexpression of three cell-cycle related genes, *Mdm2, Cdk4* and *Irf1*.

MATERIALS AND METHODS

Cell cultures. The BALB/c 3T3 A31-1-1 cell line [12] was provided by the Japanese Cancer Research Resources Bank (Tokyo, Japan). The BALB/c 3T3 A31 cell line was obtained from the American Type Culture Collection (Rockville, U.S.A.). The cells were maintained in modified Eagle's minimum essential medium (Nissui, Tokyo, Japan) supplemented with 10% heat-inactivated fetal bovine serum, (Cytosystems, N.S.W., Australia). After inoculation at 3.0×10^5 per 100-mm tissue culture dish, they were grown at 37°C in a humidified 5% CO_2 atmosphere. Cells were irradiated with a ^{60}Co γ-irradiator at 1.07 Gy/min or 48.0 Gy/min.

Flowcytometry analysis. Cell cycle analysis was performed using a FACScan instrument (Becton Dickinson, San Jose, CA, U.S.A.) as described previously [11]. DNA synthesis was assessed as the number of bromodeoxyuridine (BrdUrd)-incorporated cells. The amount of DNA was measured by fluorescence intensity of propidium iodide. Cell cycle analysis was carried out at least twice.

Immunoprecipitation of p53. Cells were inoculated at 3×10^5 per 100-mm tissue culture dish, grown up in medium for 2 days, and then incubated in methionine-free Dulbecco's modified Eagle's minimum essential medium (Flow Lab., Irvine, UK) for 1 hr before γ-irradiation or mock-irradiation. Labeling with 50 μCi/ml [^{35}S]L-methionine (Amersham, Bucks, UK) for 1 hr followed immediately thereafter, and then the cells were harvested. After washing with phosphate-buffered saline, proteins were extracted with RIPA buffer containing 10 mM Tris-HCl (pH 7.4), 0.6 M NaCl, 0.1% sodium dodecyl sulfate, 1% Nonidet P-40, 0.1% sodium deoxycholate, 1 mM EDTA, 10 μg/ml

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Abbreviations used: BrdUrd; bromodeoxyuridine, FITC; fluorescein isothiocyanate, Irf1; interferon regulatory factor 1.

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aprotinin (Wako Pure Chemicals, Osaka, Japan) and 1 mM phenylmethylsulfonyl fluoride. Immunoprecipitation was carried out with either p246 anti-p53 antibody, Ab-4 (Oncogene Science Inc., Uniondale, NY, U.S.A.) or a nonspecific IgG_1, MOPC-21 (Sigma, St. Louis, MO, U.S.A.) as a control antibody, after preclearing the lysate with mouse IgG and goat anti-mouse IgG-Sepharose 4B (Zymed, San Francisco, CA, U.S.A.). Immunoprecipitated proteins were separated by electrophoresis on 10% sodium dodecyl sulfate-polyacrylamide gel, which were dried and analyzed for radioactivity with an image analyzer (BAS2000, Fuji Film, Tokyo, Japan).

Northern blot analysis. Total RNA was isolated using the acid-phenol method described previously [13]. 20 μg of total RNA were separated on 1% agarose-2.2 M formaldehyde gel and transferred to Duralon-UV Membranes (Stratagene, La Jolla, CA, U.S.A.). The 5′-terminus coding region of mouse *p21* cDNA [14] corresponding to nucleotides 1 to 110 was used as a probe. Briefly, sense (64 mer), 5′-ATG TCC AAT CCT GGT GAT GTC CGA CCT GTT CCG CAC AGG AGC AAA GTG TGC CGT TGT CTC TTC G-3′ and anti-sense (62 mer), 5′-ACG GCA ACA GAG AAG CCA GGG CAC CTG TCA CTC GTC AAC TCG GCA CTA ACG CTA CGC GAG TA-3′ oligonucleotides were synthesized. These two oligonucleotides (150 ng each) were denatured at 95 °C for 5 min, then annealed by incubation at 65°C for 2 min and gradually cooled down to room temperature. Then the fill-in reaction was performed using the Klenow fragment with 3000 Ci/mmol, [α-^{32}P]dCTP (Amersham). The 3.3 kb *Mdm2* mRNA was detected using a 1.5 kbp fragment of the mouse *Mdm2* cDNA probe obtained by PCR amplification. The 1.7 kb *Irf1* mRNA was detected using a 1.2 kbp fragment of mouse *Irf1* cDNA. This cDNA was isolated by PCR amplification using a mouse brain cDNA library (Clontech Lab., Inc., Palo Alto, CA, U.S.A.) as a template. After subcloning, the partial sequences of both cDNAs were determined for confirmation. Hybridization was carried out as previously described [15].

RESULTS

Cell cycle states were analyzed 12 hrs after γ-irradiation. Typically, the cell-cycle phase distribution of the mock-irradiated BALB/c 3T3 A31-1-1 clone was 23% in the G1 phase, 63% in the S phase, and 14% in the G2/M phase (left panel of Fig. 1(A)). After γ-irradiation at 8 Gy, the cell-cycle phase distribution changed to 20% in the G1 phase, 42% in the S phase, and 38% in the G2/M phase (right panel of Fig. 1(A)). The number of cells in the G1 phase did not increase, while the number of cells in the G2/M phase increased from 14% to 38%. The results thus indicated that G1 arrest was abrogated. On the other hand, substantial G2 arrest occurred as shown by the increase in G2/M percentage. The results of a irradiation dose-response study performed at 0, 1, 2, 4, 8 and 12 Gy are shown in Fig. 1(B). G1 arrest was not observed at any γ-ray dose, while G2 arrest was observed as indicated by the dose-dependent increase of the number of cells in G2/M phase. In a time-course study, the G2 arrest peak was observed at 12 hrs after 8 Gy irradiation. No G1 arrest was apparent at any time-point (data not shown).

The cell-cycle state was also analyzed in the parental BALB/c 3T3 A31 clone after 2 Gy γ-irradiation (Fig. 2). Under non-irradiated conditions, the cell-cycle phase distribution was 51% in the G1 phase, 30% in the S phase, and 19% in the G2/M phase. After γ-irradiation at 2 Gy, the cell-cycle phase distribution was 56% in the G1 phase, 23% in the S phase, and 21% in the G2/M phase. The G1 phase percentage increased with a corresponding decrease in the S-phase percentage. Thus the γ-irradiation induced G1 arrest. Comparison of the cell-cycle state in non-irradiated A31 and A31-1-1 clones revealed a much lower G1 phase percentage in the latter. The doubling-times were 30 hr and 14 hr, respectively. Therefore, the G1 phase calculated for the A31-1-1 clone, 3.4 hr, was very short as compared to that in the A31 clone of 15.3 hr. The S phase length for both clones was about 9 hr, while the A31-1-1 clone showed a relatively short G2 phase length, 1.1 hr, as compared to that in the A31 clone of 5.7 hr.

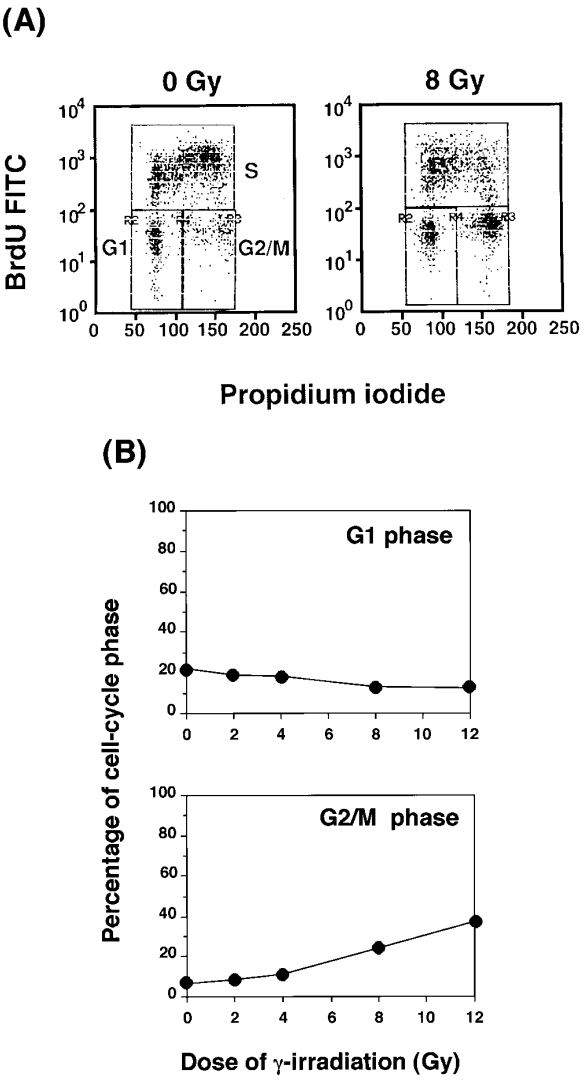

FIG. 1. Cell cycle distributions after γ-irradiation in the BALB/c 3T3 A31-1-1 clone. (A) Cell cycle distributions at 12 hrs after 0 Gy (left panel) or 8 Gy (right panel). FACScan analysis was performed by double staining. The vertical line shows the level of DNA synthesis by incorporation of BrdUrd. The horizontal line shows DNA content revealed by staining with propidium iodide. (B) The γ-ray dose-response was studied 12 hrs after irradiation.

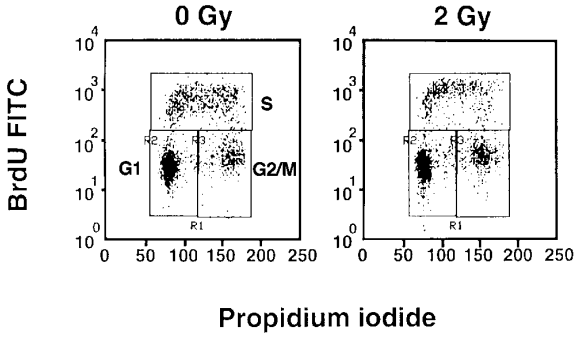

FIG. 2. Cell cycle distributions in the BALB/c 3T3 A31 clone at 12 hrs after 0 Gy (left panel) or 2 Gy (right panel) γ-irradiation. FACScan analysis was performed as described in the legend of Fig. 1.

To cast light on alterations of cellular responses associated with the abrogation of G1 arrest in A31-1-1 clone, we first analyzed p53 stabilization levels by metabolic-labeling of newly synthesized proteins following γ-irradiation. At 1 hr after γ-irradiation at 8 Gy, the p53 protein levels increased about 2-fold (Fig. 3 (A)). This increase was significant as compared to the level in C3D2F1 3T3-a cells during G1 arrest following γ-irradiation [22], indicating that p53 stabilization occurred in A31-1-1 clone. Furthermore, the level of *p21* mRNA transiently was increased about 5-fold at 2 hrs after 8 Gy irradiation (Fig. 3 (B)). These results suggested the normal function of p53-p21 pathway after DNA damage in the A31-1-1 clone.

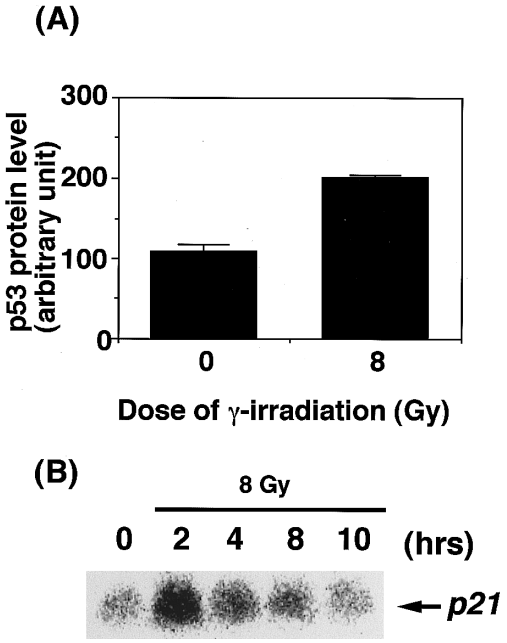

FIG. 3. p53 and p21 responses in BALB/c 3T3 A31-1-1 cells. (A) p53 stabilization after 8 Gy irradiation was analyzed. The cells were labeled with [^{35}S]-methionine for 1hr following γ-irradiation. p53 protein level was determined by measuring the radioactivity of the immunoprecipitated band. The histograms show the mean of four independent samples. (B) Northern blotting analysis of *p21* mRNA expression.

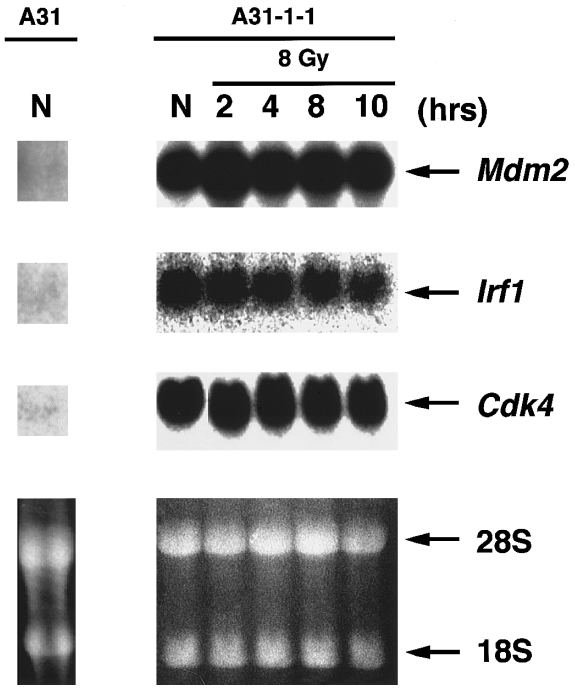

FIG. 4. Northern blotting analysis of *Mdm2, Irf1,* and *Cdk4* mRNA expression following 8 Gy irradiation in BALB/c 3T3 A31-1-1 cells. For comparison, northern blotting analysis of parental BALB/c 3T3 A31 cells are shown. N; non-irradiated cells. Equal loading was confirmed by the examination of expression level of *G3pdh* mRNA (data not shown).

Next, we determined the mRNA levels of other cell-cycle checkpoint related genes, *Mdm2, Cdk4* and *Irf1* (Fig. 4). We found that constitutive overexpression of *Mdm2* and *Cdk4* mRNAs took place as compared to the parental A31 cells. No further up-regulation of *Mdm2* and *Cdk4* mRNA were observed following γ-irradiation. Moreover, we found that the mRNA of *Irf1*, a putative transcription factor which regulates p53-independent signal transduction following DNA damage [21], was constitutively overexpressed. Compared to the parental A31 clone, basal levels of *Mdm2, Cdk4* and *Irf1* mRNA were respectively 90-, 16- and 9-fold increased in the A31-1-1 clone.

DISCUSSION

To elucidate factors involved in cell-cycle checkpoint mechanisms after DNA damage, we screened cell lines showing alteration of cell-cycle arrest following γ-irradiation. As presented in this study, BALB/c 3T3 A31-1-1 cells lacked G1 arrest. This clone was isolated as a variant of BALB/c 3T3 A31 cells by Kakunaga and Crow [12]. A31-1-1 clone shows a high frequency of

transformation after DNA damage induced by UV-irradiation or alkylating agents such as *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG) as well as a high incidence of tumorigenesis in nude mice [16,17,18]. It has therefore been frequently used for transformation studies. It is possible that the G1 arrest abrogation following DNA damage contributes to transformation or tumorigenicity in the A31-1-1 clone.

To identify the responsive factors involved in abrogation of observed G1 arrest, we first examined the p53-p21 pathway. Both p53 stabilization and induction of *p21* mRNA were observed to take place, indicating that the p53-dependent pathway does function in the A31-1-1 clone. Therefore, we concluded that abrogation of G1 arrest was caused by alteration of downstream processes of the *p21* mRNA induction in the p53-dependent pathway or by the alteration of the p53-independent pathway. The fact that the mRNAs for the cell-cycle related *Mdm2, Cdk4* and *Irf1* were constitutively overexpressed in A31-1-1 clone indicated this possibility. Mdm2 is known as a feedback regulator of p53 which is induced transiently following DNA damage [19]. Its overexpression has been reported to cause abolishment of G1 arrest following γ-irradiation [8], and thus it might have been involved in the A31-1-1 clone in the abrogation of G1 arrest. However the observed p53 stabilization and *p21* mRNA induction argues against this. Barak, Y. *et al.* similarly reported that in the mouse BALB/c cell line DM3, *Mdm2* mRNA is overexpressed [20], but showed that the possesion of longer 5'-terminal sequences compared to the one induced by DNA damage, resulted in a Mdm2 protein lacking the ability to bind p53. Therefore, it is also possible that the Mdm2 protein overexpressed in the A31-1-1 clone might not possess p53 binding and concealing activity.

Cdk4 tyrosine phosphorylation was recently reported to be involved in G1-arrest following UV-irradiation [9]. Although *Cdk4* mRNA is known to be constitutively expressed through the cell-cycle, constitutive overexpression has been described for several cancer cell-lines [21-23]. Such overexpression of *Cdk4* might promote G1 to S phase progression, and contribute for the abrogation of G1 arrest.

Irf1 has been found to be a transcription activator of interferon-inducible genes which functions in a growth-suppressive manner. *Irf1* is able to suppress transformation by *c-Ha-Ras*, c-*Myc* or *FosB* [24-25] and DNA-damage induced G1 arrest has been shown to be abrogated in *Irf1*$^{-/-}$ murine fibroblast cells [10]. DNA-damage induced apoptosis is also lost in *Irf1*$^{-/-}$ spleen-derived mature T-lymphocyte [26]. Thus, Irf1 might be a key factor in *p53*-independent DNA-damage response pathways. The constitutive overexpression of *Irf1* mRNA in the A31-1-1 clone would therefore not be expected to contribute to the abrogation of G1 arrest. Instead, the overexpression of this growth-suppressive *Irf1* gene might be considered as one of the feedback responses of the A31-1-1 clone, which showed a growth-enhanced state through the G1-S transition. Its exact role remains to be clarified.

We also noted drastic shortening of the G1 phase in the A31-1-1 as compared to the A31 clone under non-irradiated conditions. This could be due to the constitutive overexpression of *Mdm2* and *Cdk4* mRNAs. It is also possible that this shortening of the G1 phase might result in the omission of one or more steps which are required for G1 arrest induction after DNA damage.

In conclusion, although the mechanisms of G1 arrest abrogation in BALB/c 3T3 A31-1-1 cells could not be determined precisely in the present study, we demonstrated that in spite of the existence of normal p53 and p21 responses, the loss of G1 arrest occurred due to the abnormal regulation of other cellular factors. Identifying the responsible factors will contribute to elucidation of the cell-cycle controlling mechanisms.

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