

X-rays Induce Dose-dependent and Cell Cycle-independent Accumulation of p21^{sdi1/WAF1}

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ABSTRACT

Cell cycle arrest at the G1 checkpoint is governed by a function of wild-type *p53*. We assessed the behavior of the *sdi1* gene, which codes for a 21kDa potent inhibitor of cdk/cyclins, after X-irradiation. X-irradiation induced *sdi1* mRNA accumulation and G1 arrest only in cells possessing wild-type *p53*. Elevation of p21^{sdi1/WAF1} was preceded by p53 accumulation, which occurred despite *p53* mRNA constancy in normal cells growing in the log phase. The quantity of accumulated p53 and p21^{sdi1/WAF1} was radiation dose dependent. A decrease in the S phase cell population in normal cells observed after irradiation reached a minimum at less-than-maximum levels of p53 and p21^{sdi1/WAF1}. Furthermore, an accumulation of p53 and p21^{sdi1/WAF1} was also observed when cells were synchronized in the G0, G1 and S phase and X-irradiated. These results indicated that an X-ray induced p53 and p21^{sdi1/WAF1} accumulation mechanism exists throughout the cell cycle, and that the signal strength induced by X-irradiation is dose-dependent.

Key words: X-irradiation, p21^{sdi1/WAF1}

Various DNA damaging agents such as ionizing radiation, ultraviolet (UV) light, or chemical mutagens can cause transient cell cycle arrest in many types of cells. Growth arrest at the G1 or G2 checkpoints presumably prevents damaged cells from proceeding into the S and M phases, respectively, where the damaged DNA would be fixed and/or propagated. It is known that the tumor suppressor gene *p53* plays a key role in G1 checkpoint control. Dysfunction of this gene results in the loss of checkpoint arrest¹³⁾. In normal cells, p53 rapidly accumulates in the nuclei after DNA damage and works as a transcription factor to transactivate a number of genes with p53 binding sequences, such as *mdm2*⁴⁾ and *gadd45*¹²⁾. Some of these p53 inducible genes are thought to act as growth suppressors following G1 arrest.

There are cumulative data showing that complexes of cyclin dependent kinases (cdk) and cyclins are largely responsible for cell cycle progression in eukaryotic cells¹⁸⁾. Irregular regulation on cdk/cyclins is characteristic of several types of cancers, and abnormalities of these cdk/cyclin inhibitors have been thought to be one mechanism behind this deregulation⁹⁾. Among these inhibitors, a p53 inducible factor, known by several names,

sdi1/WAF1/cip1/CAP20, coding for a 21 kDa protein, has been cloned in several laboratories^{7,8,15,22)}. To elucidate the mechanism leading from DNA damage through p53 accumulation to the checkpoint arrest, we assessed the inducibility and temporal kinetics of *sdi1/WAF1* mRNA and protein after X-irradiation in logarithmically growing cells and synchronized cells. We present here data that *sdi1/WAF1* mRNA and protein (p21^{sdi1/WAF1}) accumulated in parallel after X-irradiation. Furthermore, p21^{sdi1/WAF1} and p53 accumulated in a dose-dependent manner and cell cycle-independently.

MATERIALS AND METHODS

Cells. Human primary fetal skin fibroblasts (F03), human fetal lung fibroblasts (TIG-3), undifferentiated thyroid carcinoma (8505C)¹⁰⁾, colon carcinoma (SW837), and SV40 immortalized fibroblasts (WI38VA13) were cultured with α -MEM (Gibco BRL, Gaithersburg, MD) and promyelocytic leukemia (HL60) and erythroleukemia (K562) cells with RPMI 1640 (Nikken, Tokyo). The media used were supplemented with 10% fetal bovine serum: FBS (Intergen, NY), 100 unit/ml penicillin G and 100

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µg/ml streptomycin (Gibco BRL). Cells used in these experiments were in log-phase growth at 30–50 % confluence in order to avoid *sdi1/WAF1* accumulation as a result of contact inhibition, especially in primary cells. Synchronization of TIG-3 cells was achieved by culturing in a medium containing 0.5% FBS for 3 days, and reentry into the cell cycle was achieved by raising the FBS concentration to 10%.

X-irradiation. Cells growing in log phase or synchronized were irradiated by an X-ray generator (WSI-250S; Shimadzu, Kyoto) operated at 220 kVp, 8 mA with 0.5 mm Al and 0.3 mm Cu filters at the dose rate of 0.234 Gy/min.

RNA analysis. Cells were collected at various times after irradiation. Data shown as "0" indicate mock treated cells. Total RNA was recovered by TRIZol reagent (Gibco BRL). Northern blotting was performed using 10 µg of RNA according to a previously described protocol¹⁶. *sdi1* cDNA probe was excised from BamHI digested pCDRα-*sdi1*¹⁵. *p53* cDNA probe was obtained from BamHI digested pC53-SN [The kind gift of Dr. Bert Vogelstein (Johns Hopkins University, MD)]. Probes for *histone H3* and *cyclin D1* were PCR amplified cDNA from TIG-3 poly A RNA using the following primer pairs: 5'-GGTTGGTGGTCTGACTCTAT-3' and 5'-GGATTGTCTTGGGCATAATA-3', 5'-GGATGTCTTTGGGCATAATA-3' and 5'-GCGTGTGAGCGCGTAGTAGG-3', respectively.

Western analysis. Cells were lysed with EBC buffer [10 mM Tris-HCl (pH 7.4), 125 mM NaCl, 1 mM EDTA, 1 % NP-40] containing 10 µg/ml leupeptin, 5 µg/ml aprotinin, 100 µg/ml PMSF, 100 mM NaF and 20 µM sodium orthovanadate. Equivalent protein from each sample, as measured by a protein assay kit (Bio-Rad, Hercules, CA), was electrophoresed on an SDS polyacrylamide-gel and blotted onto PVDF membrane. The membrane was blocked with 5% skim milk, hybridized with monoclonal antibodies, pAb1801 for p53 and Ab-1 for p21^{sdi1/WAF1} (Oncogene Sci., Cambridge, MA), and detected according to the protocol accompanying the ECL™-kit (Amersham, Arlington, IL). Densitometric analyses were performed on digitized images of the exposed films using NIH image version 1.59 Gel plotting: macro on a Macintosh computer.

Flow cytometry. Cells were fixed with 10% formalin and subsequently with 70% ethanol and treated with 2 mg/ml RNase A. The DNA content was detected by staining with 10 µg/ml propidium iodide. The stained cells were analyzed by FACScan™ (Becton-Dickinson: Immunocytometry Systems, San Jose, CA) with the CellFIT™ program, and the data was gated according to the doublet discrimination model. The cell cycle index was calculated using the rectangle fit analysis model.

RESULTS

sdi1/WAF1 mRNA accumulation after X-irradiation.

It has been demonstrated by Kastan et al that there is a positive correlation between expression of wild-type *p53* genes in human hematopoietic cells and their ability to arrest in the G1 phase after γ -irradiation¹¹. We evaluated the change of cell cycle distribution by flow cytometry in cells possessing various *p53* genotypes at 14 hours after 2 Gy X-irradiation. In F03 with wild-type *p53*, a significant decrease of the proportion of cells in the S phase and an increase in G2/M with a constant proportion in the G0/G1 phase was observed (Fig. 1A). In contrast, cell lines that harbored dysfunctional *p53* (8505C, SW837: codon 248 mutant; K562, HL60: deletion mutant; WI38VA13: *p53* was functionally inactivated by SV40 T antigen) showed no apparent decrease S phase but rather an accumulation in the G2/M phase portion. Accordingly, we have confirmed that the wild-type *p53* function is essential for radiation induced cell cycle arrest at G1 phase but not necessary for the G2 checkpoint. We then checked the inducibility of *sdi1/WAF1* mRNA after 2 Gy X-irradiation in the same cells used in the cell cycle experiments. The temporal change of *sdi1/WAF1* and *p53* mRNA levels after X-irradiation is shown in Fig. 1B. Accumulation of *sdi1/WAF1* mRNA increased and reached a maximum at 4 hours after X-irradiation in F03, but there was no such accumulation in the cells with dysfunctional *p53* (Fig. 1B) even at higher doses (data not shown). On the other hand, the *p53* mRNA level was not altered after X-irradiation. Even in the cells carrying mutated *p53* genes, *p53* mRNA levels were not changed by X-irradiation. These results reveal that *sdi1/WAF1* mRNA accumulation after X-irradiation is closely related to the wild-type *p53* function.

Kinetics of p53 and p21^{sdi1/WAF1} accumulation.

To elucidate the kinetics of cell cycle arrest and p21^{sdi1/WAF1} expression, logarithmically growing F03 cells were X-irradiated with 2 Gy, and the cell cycle distribution was measured. Simultaneously p53 and p21^{sdi1/WAF1} accumulation was periodically analyzed by Western blotting. The proportion of S phase cells decreased gradually and reached a minimum at 12 hours, indicating inhibition of S phase entry at the G1 checkpoint. On the other hand, the G2/M fraction increased and peaked at 12 hours as a result of G2 checkpoint arrest. The fraction of cells in the G0/G1 phase was not altered (Fig. 2A). Induction of *sdi1/WAF1* mRNA, with a maximum around 4 hours (Fig.1B), was preceded by a peak p53 accumulation at 1 hour (Fig. 2B). We confirmed that the increase of p53 was controlled not at the mRNA level but at the protein level (Fig. 1B), which may result in the upregulation of p53 dependent transactivation¹⁴. Moreover, *sdi1/WAF1* mRNA was also induced in

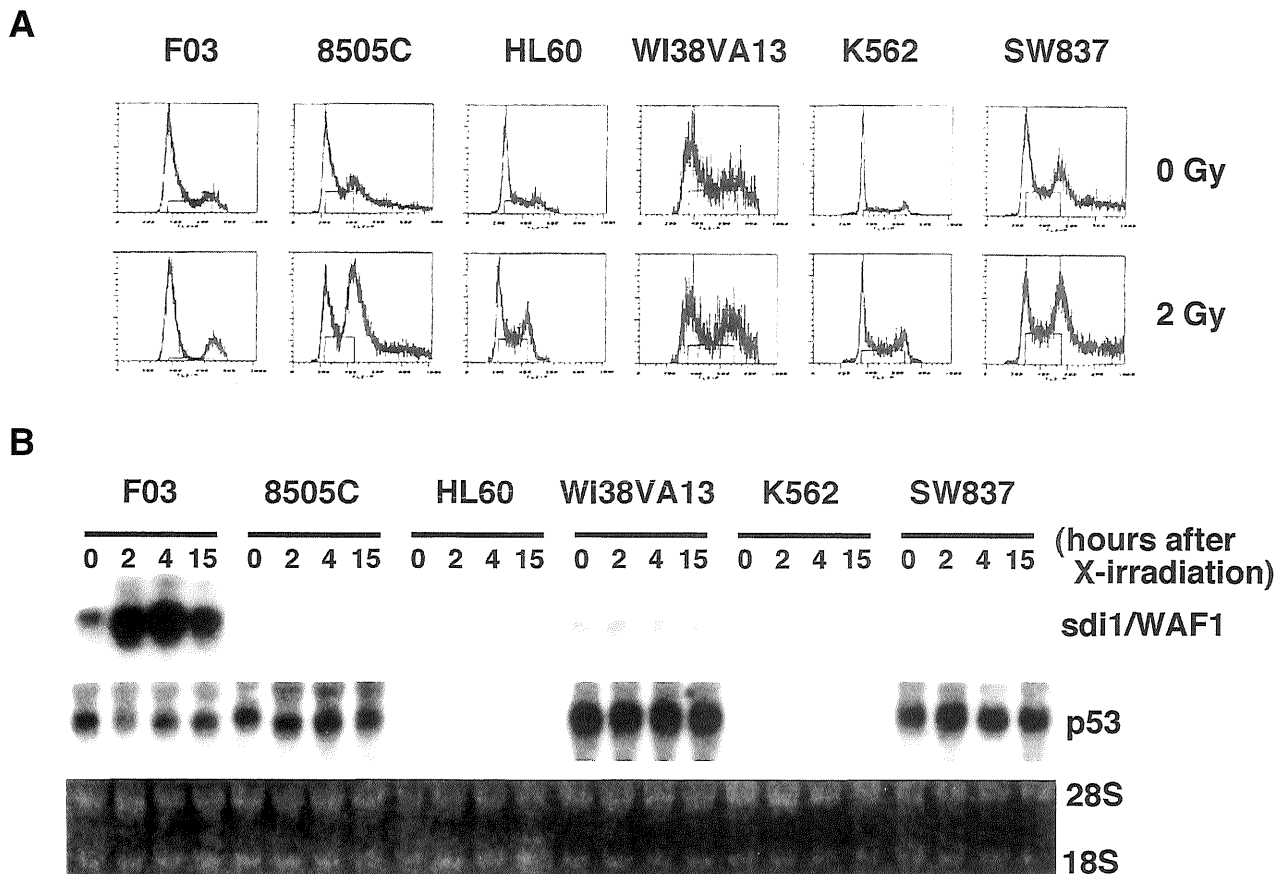


Fig. 1. Dependence of cell cycle arrest and *sdi1/WAF1* mRNA accumulation on wild-type *p53* after X-irradiation. A. F03, 8505C, HL60, WI38VA13, K562, SW837 cells growing in log-phase were 2 Gy X-irradiated or sham treated and recovered after 14 hours. About 20,000 fixed and stained cells were used for flow cytometry. The vertical axis indicates the relative number of events and the horizontal axis shows the relative fluorescence intensity, which indicates the DNA content. The small square in each histogram shows the region corresponding to the S phase. B. Total RNA was recovered after 2, 4 and 15 hours from 2 Gy X-irradiated cells; the zero-hour RNA was from untreated cells. 10 μ g total RNA was used for Northern blotting of *sdi1/WAF1* and *p53* mRNA. The lower photograph shows 28S rRNA stained with ethidium bromide.

the same way because a *p53* binding sequence exists in its proximal promoter sequence⁷. Interestingly, a temporary drop in the *p53* level observed at 4 hours (Fig. 2B) was reproducible even using another anti-*p53* antibody. However, the mechanism and meaning of this phenomenon are not clear. *p21^{sdi1/WAF1}* accumulation started in concert with *p53* accumulation at 1 hour and reached a peak at 4 hours, then gradually decreased although it was sustained above initial levels after more than 12 hours (Fig. 2B). The accumulation of *p21^{sdi1/WAF1}* correlated with mRNA levels but not with *p53* levels. Stabilization of *sdi1/WAF1* mRNA might occur irrespective of subsequent *p53* levels once its transcription is activated by the *p53* at around 1 hour.

Dose dependent accumulation of *p53* and *p21^{sdi1/WAF1}*. In addition, because the correlation between X-ray dose and *p53* and *p21^{sdi1/WAF1}* levels is not well understood, we measured *p53* and *p21^{sdi1/WAF1}* accumulation by Western blotting following graded doses of radiation (0, 0.5, 1, 2, 5, 10

Gy) in logarithmically growing F03 cells. And accumulation of *p53* and *p21^{sdi1/WAF1}* was observed after irradiation at doses as low as 0.5 Gy (Fig. 3A and B), and *p53* increased dose dependently. *p21^{sdi1/WAF1}* increased in a dose dependent manner up to 2 Gy and reached a plateau at doses over 2 Gy. Simultaneously, the proportion of S phase cells was measured by flow cytometry. The S phase cells decreased and approached a minimum with 2 Gy. The proportion of S phase cells reached a minimum level following further higher doses, as in our observations on *p21^{sdi1/WAF1}* kinetics. It is interesting that less-than-maximal levels of *p53* are adequate for maximal G1 arrest.

Induction of *p53* and *p21^{sdi1/WAF1}* accumulation in G0, G1 and S phase cells. It is possible that the induction mechanism of *p53* and *WAF1* exists only in the G1 phase, because the above data using logarithmically growing cells indicate that the *p53*-*WAF1* system functions only in the G1 checkpoint mechanism. To clarify this, we synchronized TIG-3 cells and X-irradiated them at the

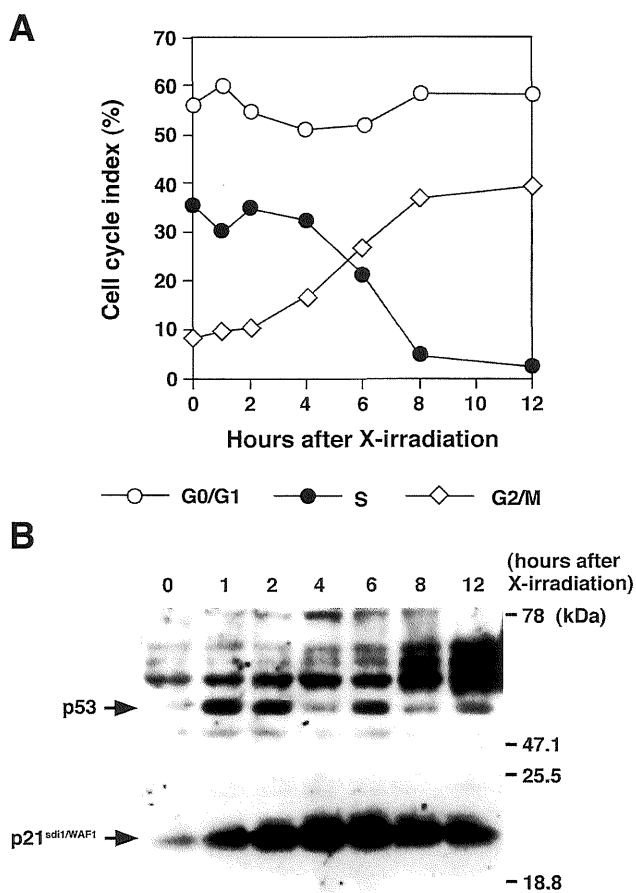


Fig. 2. Cell cycle distribution and temporal alteration of p53 and p21^{sd11/WAF1} after 2 Gy X-irradiation in F03 cells.

Cells were 2 Gy X-irradiated and recovered after 1, 2, 4, 6, 8, 12 hours. 0 shows non-irradiated cells. A. Cells were fixed and analyzed by flow cytometry. Cell cycle index was calculated with a rectangle fit model. B. Total protein was recovered and subjected to Western blotting probed with anti-p53 and anti-p21^{sd11/WAF1} monoclonal antibodies.

G0, G1, or S phases. TIG-3 cells were synchronized at the G0 phase by serum starvation for 3 days, then stimulated to enter a new cell cycle by reintroduction of serum. Cell cycle synchronization and re-entrance into the cell cycle was confirmed by [³H]-thymidine incorporation and expression of marker genes. TIG-3 cells began to synthesize DNA between 12 and 18 hours indicating S phase entry (Fig. 4A). By Northern blot analysis, the accumulation of cyclin D1 in the mid G1 phase at 8 hours after serum stimulation, and histone H3 at the G1/S boundary at 12 hours, were confirmed (data not shown). Therefore, serum deprived cells (G0 cells), cells at 8 hours after serum stimulation (cells in the G1 phase), and cells at 21 hours after stimulation (cells in the S phase) were X-irradiated with 2 Gy and collected 1 hour and 4 hours later. As shown in Fig. 4B, more p53 and p21^{sd11/WAF1} was observed in X-irradiated cells compared to non-irradiated control in every phase examined. These data suggest that

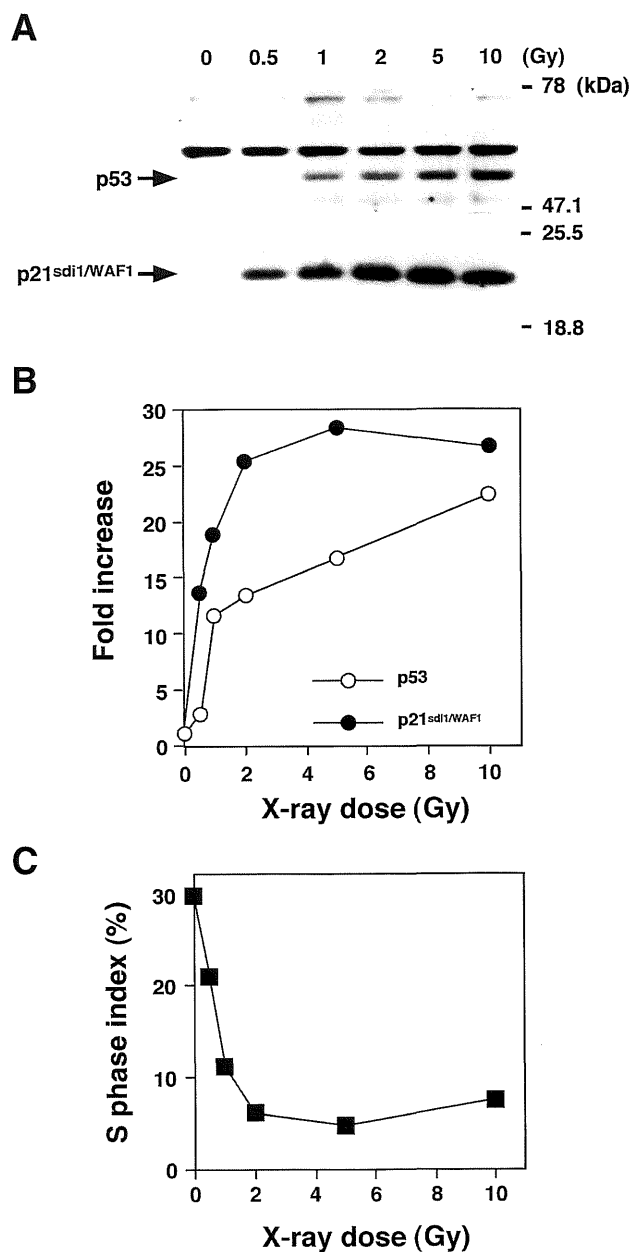


Fig. 3. X-ray dose dependent p53 and p21^{sd11/WAF1} accumulation and cell cycle arrest in X-irradiated F03 cells.

Cells growing in logarithmic phase were X-irradiated at 0, 0.5, 1, 2, 5, 10 Gy.

A. Western blotting was performed using cells at 1 hour for p53 analysis, and at 4 hours for p21^{sd11/WAF1} analysis after X-irradiation. B. Intensity of p53 and p21^{sd11/WAF1} bands was measured and relative intensities compared to 0 Gy bands are plotted. C. Fixed cells at 14 hours after X-irradiation were analyzed by flow cytometry, and the S phase index is plotted against X-irradiation dose.

the induction mechanism of p53 and p21^{sd11/WAF1} exists throughout the cell cycle.

DISCUSSION

Recently, it has become clear that cdk/cyclin pairs drive the cell cycle transition *sd11/WAF1* machinery. We have presented supportive data

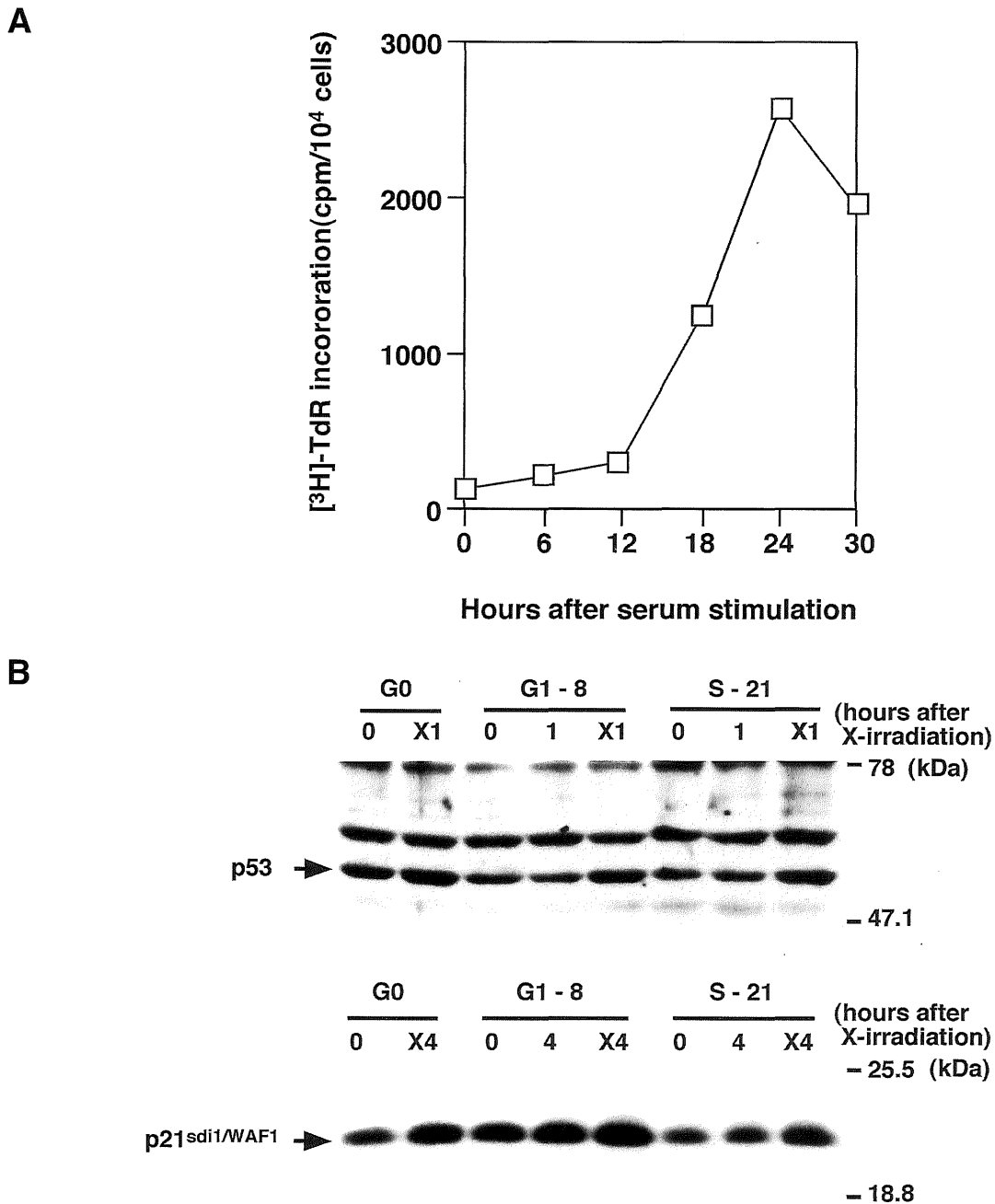


Fig. 4. Accumulation of p53 and p21^{sdi1/WAF1} in synchronized cells at G0, G1 and S phases in F03 cells. A. DNA synthesis was measured by [³H]-thymidine pulse label at the indicated times after serum stimulation. B. G0 cells, G1 cells (8 hours after serum stimulation) and S cells (21 hours after serum stimulation) (all lanes labeled with a "0") were 2 Gy X-irradiated (indicated as "X1 or X4") or unirradiated (1 or 4), and recovered for Western blotting 1 hour later for p53 analysis and 4 hours later for p21^{sdi1/WAF1} analysis.

here using X-irradiation to show that p21^{sdi1/WAF1} negatively regulates these cdk/cyclin complexes and acts as a downstream effector of p53 following γ -irradiation⁶. Inhibition of cdk/cyclins cause underphosphorylation of RB protein and G1 arrest²⁰. Deng et al demonstrated that *sdi1/WAF1* was essential for G1 checkpoint arrest because *sdi1/WAF1* knock-out cells lost their G1 checkpoint after irradiation⁵. In our experiments, cells carrying not only knock-out but also mutated p53 did not show G1 cell arrest but did show G2 cell arrest after X-irradiation. On the other hand, the

observation in our study that the p53-p21^{sdi1/WAF1} induction mechanism exists in the S phase raises the possibility that it plays an active role in every phase. In fact, forced expression of wild-type p53 in a p53 deficient cell line resulted in G2 arrests¹ and p21^{sdi1/WAF1} inhibition of G2 cdk/cyclins as well as G1 cdk/cyclins²². Accordingly, p53-p21^{sdi1/WAF1} may work as part of the G2 checkpoint machinery although, as we discussed above, it is not essential for the arrest mechanism. On the other hand, it is possible that accumulated p53 and p21^{sdi1/WAF1} perform other functions by binding directly or indi-

rectly to several proteins. p53 binds to many cellular proteins which have roles in transcription, replication and repair¹⁹, while p21^{sd1/WAF1} interacts with PCNA (proliferating cell nuclear antigen) to activate repair and to suppress replication¹⁹. X-ray induced p53 and p21^{sd1/WAF1} in G0, G1 and S might perform these roles. Meanwhile, the accumulation of p53 for cell cycle arrest following over 2 Gy may suggest that p53 is required for the repair of damaged DNA.

It is interesting that the p53 level oscillated after X-irradiation. We hypothesized that the radiosensitivity differed among cells with different p53 levels, but the colony forming efficiencies were not altered when the cells were exposed to an additional 2 Gy X-irradiation at several points where the p53 levels differed (data not shown). Considering that p53 is often referred to as the "guardian of the genome", mutation frequency may differ according to p53 levels. Alternatively, it is possible that the function of accumulated p53 differs between the first peak at 1 hour and the second peak at 6 hours. As discussed above, p53 may function differently at different times after radiation insult.

The upstream mechanism that induces p53 accumulation is gradually becoming clear. The dose dependent increase of p53 indicates the existence of a dose dependent upstream signal. Several enzymes are known to be activated by ionizing radiation. In cells from A-T (ataxia telangiectasia) patients, p53 and p21^{sd1/WAF1} accumulation after irradiation was much lower than in normal cells². Thus, ATM (ataxia telangiectasia mutated), a candidate gene responsible for A-T¹⁷, works between the primary signal and p53 accumulation. Indeed, ATM protein acts as a protein kinase and phosphorylates p53 after DNA damage^{3,12}. Similarly, other kinases are reported to phosphorylate p53²¹. However, the question which is the key enzyme of p53 activation after X-irradiation remains to be elucidated.

Taken together, our data suggest a dose dependent p53-p21^{sd1/WAF1} induction machinery throughout the cell cycle which results in checkpoint arrest and possibly other cellular activities such as repair and replication.

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