Expression of Ribonucleotide Reductase after Ionizing Radiation in Human Cervical Carcinoma Cells

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ABSTRACT

Ribonucleotide reductase (RR), the rate-limiting enzyme in the de novo synthesis of deoxynucleotide triphosphates (dNTPs), is a potential target for cancer therapy. We characterized the response of RR in a human cervical carcinoma cell line, Caski, after damage by ionizing radiation (IR). We also investigated the cell cycle regulation of both the regulatory (R1) and catalytic (R2) RR subunits in an attempt to distinguish between a direct DNA damage induction of RR by IR and a cell cycle-dependent expression of RR after IR. Confluently, growth-arrested Caski cells showed a ≥5-fold increase in R2 mRNA and an 18-fold increase in R2 protein as cells entered S phase after serum stimulation. The R2 protein levels peaked in late S phase and returned to lower basal levels in G0-M. No changes in R1 mRNA and protein levels occurred with progression through the cell cycle after serum stimulation. In growth-arrested Caski cells treated with IR (6 Gy) without serum stimulation, a similar rise (17-fold) in R2 protein was evident at 24 h after IR and was associated with a 4-fold increase in situ RR enzyme activity, but no increases in R1 and R2 mRNA nor R1 protein were found. E2 promoter binding factor 1 mRNA and protein levels also showed no change after IR. Growth-arrested controls (no IR and no serum stimulation) showed <4-fold elevation in R2 protein. These data suggest that RR plays a role in IR-mediated damage responses in Caski cells, which appears different than RR regulation after proliferation (serum) stimulus. Such a response to IR in human tumor cells has not been reported previously. The use of specific R2 protein or RR enzyme inhibitors after IR may enhance IR cytotoxicity by altering this potential RR-mediated repair pathway.

INTRODUCTION

Sufficient dNTP levels are essential for both DNA replication and DNA repair synthesis including excision repair, recombinational repair, and postreplication repair (1). Additionally, the relative ratios among dNTPs must be maintained to ensure high fidelity for both types of DNA synthesis (2). Therefore, enzymes involved in the biosynthesis of dNTPs might be expected to be highly regulated during S phase and after DNA damage to prevent dNTP misincorporation and to decrease mutation frequency.

RR is the rate-limiting enzyme in the de novo synthesis of all dNTPs. RR contains two identical large regulatory subunits, R1, and two identical small catalytic subunits, R2 (3). RR holoenzyme activity is activated in S phase. Using a variety of methods to synchronize cells at different stages in the cell cycle, the mRNA and protein levels of R1 and R2, as well as holoenzyme activity level, have been measured in several systems including Escherichia coli and yeast and mammalian cells. In most mammalian cells, steady-state mRNA levels for both R1 and R2 are low in G0-G1, rise at the G1-S border, and decline in G2-M (4-7). In general, R2 mRNA levels undergo a more dramatic change during the G1-S-phase transition, whereas R1 mRNA levels show only a modest increase during S phase (4-7). The protein levels of R1 and R2 during different stages of the cell cycle have also been measured either by quantification of purified R1 and R2 proteins using an in vitro enzymatic activity assay or by electron paramagnetic resonance measurement of the tyrosyl radical in the R2 subunit.

The experimental data on regulation of R1 and R2 protein levels in mammalian cells are somewhat conflicting. For example, it was found in mouse S49 T lymphoma and mammary tumor cells that R1 protein levels were relatively constant throughout the cell cycle, whereas the R2 protein level was higher in S phase compared with its level in G1 phase (8-10). Both R1 and R2 protein levels were, however, low in quiescent (G0) cells (11, 12). These studies suggested that R2 protein levels were rate limiting and controlled the level of RR enzyme activity during DNA replication. Other studies using mouse Ehrlich tumor cells and regenerating rat liver indicated that although the R2 protein level was higher during DNA replication, it may be present in excess (13, 14). It was consequently determined that the R1 protein was the rate-limiting factor for RR enzyme activity in these mammalian cells (13, 14).

Additionally, it has been reported by several groups that various types of DNA-damaging agents including UV light, HU, and alkylating agents can induce RR in bacteria, yeast, and several different mammalian cell systems including mouse Balb/3T3 fibroblasts and HU-resistant cells derived from the mouse mammary tumor cell line, TA3 (15-25). These data suggest that the induction of RR may facilitate DNA repair after treatment with these DNA-damaging agents. For example, nrdA and nrdB genes encode both the B1 RR regulatory subunit and the B2 catalytic subunit in E. coli. Treatment with 50 mM HU enhanced the transcription of both nrdA and nrdB genes from the nrdA promoter, whereas transcription was reduced in SOS mutants, suggesting that the elevation of RR expression was induced by a HU-mediated DNA damage signal (15). In Saccharomyces cerevisiae, the RNR1 and RNR3 genes encode the large subunit, whereas the RNR2 gene encodes the small subunit. Under normal growth conditions, only RNR1 and RNR2 are expressed. However, in response to DNA-damaging agents such as 4-NQO, MMS, UV light, and HU, RNR3 was induced (19, 23), and the transcription of both RNR1 and RNR2 was enhanced (19-22, 24, 25). In mouse BALB/c 3T3 cells, RR activity was induced by treatment with an alkylating agent, chlorambucil, and accompanied by increases of R1 and R2 mRNAs and R2 protein levels (26). Additionally, the promoters of R1 and R2 were activated after UV irradiation in the same mammalian cells (27).

The regulation of RR after DNA damage by IR has not been studied. We reported recently, however, that a post-IR exposure to the RR inhibitor, HU, resulted in significantly greater radiosensitization in human Caski cervical carcinoma cells compared with a modest radiosensitizing effect in these human tumor cells after a pre-IR exposure to HU (28). Our results, using an univariate analysis of the α and β parameters from the linear-quadratic model of IR damage/repair, suggested that a post-IR exposure to HU may convert IR-induced repairable DNA lesions into irreversible DNA lesions (28).

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3 The abbreviations used are: dNTP, deoxynucleotide triphosphate; RR, ribonucleotide reductase; HU, hydroxyurea; 4-NQO, 4-nitroquinoline 1-oxide; IR, ionizing radiation; MMS, methylmethanesulfonate; PVDF, polyvinylidene difluoride; BrdUrd, bromodeoxyuridine; PBS, fetal bovine serum; PI, propidium iodide; E2F1, E2 promoter binding factor 1.

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Additionally, other investigations have found that HU treatment immediately after IR directly inhibited IR-induced DNA repair in HeLa cells using alkaline elution techniques (29). These studies imply that RR may be involved in IR-induced DNA repair processes. One hypothesis for the role of RR in IR-damage repair is that RR subunits are elevated after IR in Caski cells, resulting in an increase of dNTP pools, which facilitate DNA repair.

In this study, the RNA and protein levels of both the R1 and R2 subunits in Caski cells were measured after IR in both exponentially growing cells and in confluent and growth-arrested cells. We also investigated the cell cycle regulation of both subunits in Caski cells in an attempt to distinguish between a direct DNA damage induction of RR and a cell cycle-dependent expression of RR after IR.

**MATERIALS AND METHODS**

**Cell Culture.** Caski cells were cultured in RPMI 1640 (Grand Island, NY) supplemented with 10% fetal bovine serum and 25 mM HEPES buffer (complete medium). Caski cells were obtained from the American Type Culture Collection (Rockville, Maryland; Ref. 30). Caski cells have a population doubling time of 19 h and a plating efficiency of 30%. Caski cells contain human papillomavirus type 16 and express the E6 protein (31). These cells contain the wild-type p53 gene and express p53 mRNA but do not express RR (Ref. 31).

**Synchronization of Cells.** Caski cells were cultured in complete medium until confluence was achieved. Iodine was replaced every day for 5 days, after which cells were contact inhibited. Cells were then further serum-arrested using growth in medium containing 0.2% FBS (low-serum medium) for an additional 2 days. Cells were then treated with or without IR in low serum medium or replated in complete medium without prior irradiation.

**Irradiation.** Cells were irradiated using a 137Cs radiation unit at a dose rate of 5.9 Gy/min.

**Northern Blot Hybridization.** Total RNA was isolated by RNeasy (Tel-Test, Inc., Friendswood, TX) as described in the manufacturer’s protocol. Total RNA (20 μg) from each sample was then fractionated by electrophoresis on a formaldehyde agarose gel using a standard technique (34). RNA samples were transferred to nylon membranes (Micron Separations, Inc., Westborough, MA) using a pressure blotter (Stratagene, La Jolla, CA) with 20× SSC (3 M NaCl, 0.34 M sodium citrate, pH 7.0). An EcoRI fragment from the human R2 (hR2) cDNA (provided by Dr. Yun Yen, City of Hope National Medical Center, Los Angeles, CA), a 0.5-kb BamHI fragment from the human R1 (hR1) cDNA (provided by Bio-Mega/Boehringer Ingelheim Research, Inc., Laval, Quebec, Canada), a PstI fragment from 36B4 cDNA (35, 36), and a 0.4-kb BglII/Sall fragment from human E2F1 cDNA (provided by Dr. Peggy J. Farnham, University of Wisconsin-Madison, Madison, WI) were used as probes and labeled with 32P using a random prime kit (Boehringer Mannheim Corp., Indianapolis, IN) for hybridization by standard methods (34). Membranes were probed, washed, and exposed to X-ray film (Fuji Photo Film Co., Ltd., Tokyo, Japan) and also scanned by PhosphorI mage (Molecular Dynamics, Sunnyvale, CA) analyses for quantification. The same membranes were hybridized with a [32P]cDNA probe for qualitative analysis.

**Isolation of Whole-Cell and Nuclear Protein Extracts.** The procedure for whole-cell protein extracts was described in a recent publication from our laboratory (37). The isolation of nucleic extracts was performed using a protocol published previously (38). Briefly, cells in 100-mm Petri dishes were rinsed with cold PBS twice and removed by scraping. After centrifugation (12,000 × g for 1 min at 4°C), the cell pellet was resuspended in 1 ml of ice-cold hypotonic solution [10 mM Tris (pH 7.5), 25 mM KCl, 2 mM MgAc, and 1 mM DTT] for 15 min and passed through a 1-ml syringe with a 23-gauge needle for up to 15 times. Nuclei were collected by centrifugation (1000 × g for 5 min at 4°C), resuspended, and incubated in hypertonic solution [100 mM Tris (pH 7.5), 400 mM KCl, 2 mM MgAc, 1 mM DTT, 1 mM phenylmethylsulfonyl fluoride, and 20% (v/v) glycerol] for 10 min. Nuclear extracts were centrifuged (12,000 × g for 10 min), and the protein concentration of the supernatant was determined using Bradford assays (39).

**Western Immunoblot Analyses.** Whole-cell extracts (25 μg) or nuclear extracts (20 μg) were fractionated by 10% SDS-PAGE (34). Proteins were electrotransferred onto PVDF-Plus membranes (Micron Separations, Inc., Westborough, MA) in Tris-glycine buffer containing 20% methanol. The membranes were then blocked in 5% milk in Tris-buffered saline-Tween (TBST; 20 mM Tris, 137 mM NaCl, and 0.1% Tween 20, pH 7.6) for 1 h and washed with TBST. Membranes were first incubated with rabbit anti-human R2 serum (Ref. 37; 1:10,000 dilution in TBST), mouse anti-human R1 monoclonal antibody (1:200 dilution), or mouse anti-human E2F1 monoclonal antibody (1:1000 dilution in TBST; Santa Cruz Biotechnology, Inc., Santa Cruz, CA) for 1 h at room temperature and then washed with TBST. Membranes were then incubated with a 1:10,000 dilution of anti-rabbit IgG-alkaline phosphatase conjugate and an anti-mouse IgG-alkaline phosphatase conjugate (1:10,000 for R1 and 1:100 for E2F1; Sigma Chemical, St. Louis, MO) for 1 h at room temperature and washed. Finally, membranes were incubated with AttoPhos (Molecular Dynamics, Sunnyvale, CA) for 20 min, dried, and scanned by a FluorImager (Molecular Dynamics, Sunnyvale, CA) for quantification.

**Flow Cytometric Analysis.** BrdUrd/DNA and hR2/DNA signal events from treated or untreated cells were analyzed with the use of a Becton Dickinson FACScan flow cytometer with 15-nm excitation at 488 nm. The FITC signal was collected through a 530/30 band-pass filter on FL1, and the PI signal was analyzed through a 650 long-pass filter on FL3. The software used for acquisition and analysis was Lysys II (Ref. 11; Becton Dickinson, San Jose, CA). Only singlets were included for cell cycle distribution statistics. Doublonets were excluded by analysis of two-dimensional plots of fluorescence pulse width versus area.

**CDP Reductase in Situ Activity Assay.** This assay, described by Xie and Plunkett (41) and Tanaka et al. (42), was used with modifications. Confluent and growth-arrested Caski cells were incubated with [3H]cytidine ([2-3H]C) 100 μCi/ml, 55 μCi/mmol; Moravek Biochemicals, Inc., Brea, CA; 0.5 μCi/ml) for 10 min. To monitor production of dCTP, nucleotides were extracted with ice-cold trichloroacetic acid, and both acid-soluble and -insoluble fractions were separated by centrifugation (16,200 × g for 1 min at 4°C). Acid-soluble fractions were neutralized using a 1:10 volume of freon:amine mixture [mixture: 1:1,2-trichloro-1,2,2-trifluoroethane:tri-n-octylamine (3:9:1)], and the upper aqueous phase was saved for periodate oxidation to degrade ribonucleotides. The extracted nucleotides were then mixed with 4 mM deoxyguanosine and 40 mM NaIO4, incubated at 37°C for 5 min, and then cooled on ice. Ribonucleotides were further degraded by incubation with 1 μM rhomannose (2.03 μl/60 μl) and 4 mM dimethylamine (9 μl/60 μl) at 37°C for 30 min. dNTPs were analyzed by high-performance liquid chromatography using a Partisil 10 SAX (Whatman, Hillsboro, OR) anion exchange column (4.6 × 250 mm) with a flow rate of 2 ml/min and separated by 0.35 mM NH4H2PO4 (pH 3.0 adjusted by H3PO4) supplemented with 10% acetonitrile. The radioactivity associated with dCTP was measured with a radioactive flow detector. Acid-insoluble fractions were dissolved in 0.5% SDS and incubated at 50°C for 4 h. DNA was degraded using 1 μg of DNase-free RNase A at
RESULTS

Expression of the hRR R1 Large Subunit and R2 Small Subunit after IR in Exponentially Growing Caski Cells. Exponentially growing Caski cells were irradiated to a dose of 6 Gy. This dose was selected based on our study published recently of the in vitro interaction of IR and HU, a specific RR inhibitor, in Caski cells (28). Both total RNA and total cell extract were isolated at various times for up to 24 h after IR. Expression of R1 and R2 mRNA and their corresponding protein levels were quantified by Northern (Fig. 1) and Western (Fig. 2) blot analyses, respectively. No significant (<2-fold) differences in R1 and R2 steady-state mRNA levels were observed for up to 24 h after IR (Fig. 1). Two R2 transcripts (3.4 and 1.6 kb) were detected by Northern blot analyses. These two R2 mRNA fragments result from different lengths on the polyadenylation signals of the 3' noncoding sequences (43). However, it was also found that both of these R2 transcripts contained the R2 coding region. Thus, the levels of these R2 transcripts were pooled to determine the total level of functional R2 mRNA. 36B4 was used as an internal control, because it has been reported that 36B4 is not induced by IR (36, 44). By Western blotting, no increase in R1 protein was detected for up to 24 h after 6 Gy (Fig. 2). However, the steady-state level of the R2 protein increased by 2 h, peaked at 8–12 h (2.2-fold), and then declined.

Expression of R2 Subunit through the Cell Cycle in Exponentially Growing Cells. It has been reported that expression of the R2 subunit is cell cycle dependent in several rodent cell lines (8–10). The steady-state R2 protein level was found to be low in G0-G1 cells, increased in S phase, and returned to baseline in G2-M (8–10). The expression of the R2 protein throughout the cell cycle in Caski cells was analyzed by flow cytometry using concomitant staining of both the hR2 protein and DNA (Fig. 3). In these human tumor cells, the R2 protein level was low in G1; the level progressively increased when cells entered S phase, and peaked in late S. The R2 level decreased in G2-M, as depicted in the cell population with lower intensity of FITC.
cycle effects, the cell cycle distribution of confluent and growth-arrested Caski cells were kept in medium containing 0.2% FBS after IR. At $t = 0$, 87% of cells were in G0-G1. However, the percentage of G0-G1 cells then decreased from 6 to 18 h and plateaued at $\approx 50\%$ to 30 h. The early S-phase and late S-phase fractions increased from 6 to 18 h. At 18 h, the total S-phase fraction increased 4-fold (9–37%) after 6 Gy. A control experiment was performed using Caski cells under the same culture conditions without prior IR (data not shown). Although a small percentage of G0-G1 cells (<10%) entered early S phase due to the replacement of fresh, low-serum medium, the late S and G2-M populations were not significantly ($\pm 2\%$) altered (late S: 9% at 0 h to 11% at 18–30 h; G2-M: 4% at 0 h to 6% at 30 h) throughout the time course of the experiment. Thus, these results demonstrate that replacement of fresh, low-serum medium alone at $t = 0$ stimulated a minor percentage of cells to enter early S phase. In contrast, however, 6 Gy of IR stimulated up to 50% of the G0-G1 cells at $t = 0$ subsequently proliferate and progress through S and into G2-M phases over 30 h, even in the presence of low-serum culture conditions. Such an effect of IR on cell cycle progression in confluent and growth-arrested human tumor cells has also been reported by other groups (36, 44–46).

Alteration of RR Activity in Confluent and Growth-arrested Caski Cells after 6 Gy. To test whether the increased R2 steady-state protein levels after IR lead to an enhancement of RR holoenzyme activity in confluent and growth-arrested Caski cells, RR activity levels were measured immediately before IR ($t = 0$) and at 24 h after 6 Gy using an in situ CDP reductase assay (Fig. 7). This method measured the incorporation of RR-converted, radiolabeled cytidine into both the cellular dCTP pool and into DNA. RR at 24 h after 6 Gy was 4-fold higher than RR activity measured at 0 h immediately before IR (Fig. 7).

Expression of the R1 Large Subunit and R2 Small Subunit in Semisynchronized Caski Cells. Because we found that Caski cells entered S phase after 6 Gy in confluent and growth-arrested cells staining and 4N DNA content (800 PI; Fig. 3). The ratio of the mean values of FITC fluorescence in these three populations (G1:S:G2-M) was 1:4:2.

We have also reported that the cell cycle distribution of exponentially growing Caski cells was altered after IR (28). The proportion of G2-M cells was significantly increased, S-phase cells were slightly increased, and G1 cells were decreased. Thus, our observed increase in the R2 protein level detected by Western blot after 6 Gy IR in exponentially growing Caski cells (Fig. 2) may have resulted from a change in the cell cycle distribution after IR.

Expression of the R1 Large Subunit and the R2 Small Subunit after IR in Confluent and Serum-starved Caski Cells. To limit the effect of cell cycle redistribution after IR, confluent and serum-starved Caski cells were then used to study the expression of RR at the message and protein levels. We found that R1 and R2 mRNA levels were up-regulated after IR in growth-arrested Caski cells (Fig. 4). The message induction was 2-fold for both R1 and R2 subunits at 8 h after 6 Gy. The levels declined after 10 h and then increased after 30 h. However, the steady-state level of the R1 protein did not change for up to 30 h after IR (Fig. 5). In contrast, R2 protein levels rose at 10 h after IR, peaked (17-fold increase) at 24 h, and then decreased slightly at 30 h (Fig. 5). The results from unirradiated samples showed only a 3.5-fold increase in R2 protein after 24 h. Thus, these data suggest that the marked (17-fold compared with 3.5-fold) induction of R2 protein in these growth-arrested Caski cells was a direct response to IR.

Cell Cycle Distribution after IR in Confluent and Serum-starved Caski Cells. To further characterize the relationship of the increased R2 protein expression after IR and possible IR-related cell cycle effects, the cell cycle distribution of confluent and growth-arrested Caski cells after 6 Gy was analyzed by flow cytometry (Fig. 6). Cells were kept in medium containing 0.2% FBS after IR. At $t = 0$, 87% of cells were in G0-G1. However, the percentage of G0-G1 cells then decreased from 6 to 18 h and plateaued at $\approx 50\%$ to 30 h. The early S-phase and late S-phase fractions increased from 6 to 18 h. At 18 h, the total S-phase fraction increased 4-fold (9–37%) after 6 Gy. A control experiment was performed using Caski cells under the same culture conditions without prior IR (data not shown). Although a small percentage of G0-G1 cells (<10%) entered early S phase due to the replacement of fresh, low-serum medium, the late S and G2-M populations were not significantly ($\pm 2\%$) altered (late S: 9% at 0 h to 11% at 18–30 h; G2-M: 4% at 0 h to 6% at 30 h) throughout the time course of the experiment. Thus, these results demonstrate that replacement of fresh, low-serum medium alone at $t = 0$ stimulated a minor percentage of cells to enter early S phase. In contrast, however, 6 Gy of IR stimulated up to 50% of the G0-G1 cells at $t = 0$ subsequently proliferate and progress through S and into G2-M phases over 30 h, even in the presence of low-serum culture conditions. Such an effect of IR on cell cycle progression in confluent and growth-arrested human tumor cells has also been reported by other groups (36, 44–46).

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**DISCUSSION**

The cell cycle-dependent expression of RR has been studied in a variety of cells at the message and protein levels. However, regulation maintained in low serum conditions (Fig. 6), it was possible that the observed increase in R2 protein expression after IR may have resulted from an increase in S-phase cells. To further investigate the possibility of R2 protein induction by IR independent of cell cycle redistribution, Caski cells were synchronized at G0-G1 in low serum medium and then replated in complete medium (10% FBS) without prior IR. The expression of both R1 and R2 subunits throughout the cell cycle in these semisynchronized Caski cells at both the mRNA and protein levels were characterized. The steady-state R1 mRNA level did not demonstrate any cell cycle-dependent fluctuations (Fig. 8). However, R2 mRNA levels were increased at all time points, with a maximum increase of 5.3-fold at 24 h after replating. Western blots showed that R2 protein levels were also increased at all time points (Fig. 9). Peak R2 protein levels, 17.5-fold above control levels, were observed from 24 to 30 h after replating in complete medium. In contrast, R1 protein levels did not significantly change under these conditions.

Cell Cycle Distribution after Replating Confluent and Growth-arrested Caski Cells in Complete Medium (10% FBS). The cell cycle distribution of cells after replating in complete medium without IR was also analyzed by flow cytometry (Fig. 10). These G0-G1 cells started to enter the cell cycle at 6 h after replating, as indicated by a decrease of the G0-G1 fraction and an increase of the S-phase and G2-M fractions. The S-phase fraction reached 34% at 30 h after replating in complete (10% FBS) medium.

Expression of Human E2F1 after IR. The E2F1 protein level rises at the G1-S border and has been reported to control the expression of several genes that are required for DNA replication, such as dihydrofolate reductase, thymidine kinase, and RR (47). The possible involvement of E2F1 in X-ray-inducible responses also seemed plausible, given the recent report of thymidine kinase induction after IR (44). E2F1 mRNA and protein levels were compared in both IR-treated confluent and growth-arrested Caski cells or nonirradiated confluent cells, which were replated in complete medium (10% FBS) at low density using protocols described above. E2F1 mRNA levels were induced 2-fold at 10 h after 6 Gy of IR (Fig. 4) and showed even lower levels (<2 fold) after replating in 10% FBS medium without prior IR (Fig. 8). The E2F1 nuclear protein level was also slightly increased 10–12 h (2.1-fold) after 6 Gy (Fig. 5). When cells were replated in medium containing 10% FBS without prior IR, the E2F1 protein level increased 1.9–4.6-fold throughout the course of the experiment (Fig. 9). Thus, the relative nuclear E2F1 protein levels were higher when the cells were stimulated into S phase by replating in complete media at low density without IR compared to the levels induced by IR, although both conditions had comparable early S-phase fractions (Figs. 6 and 10).
of RR throughout the cell cycle in human cells has not been reported previously. As part of this study, the message and protein levels of both the R1 and R2 subunits were characterized throughout the cell cycle in Caski cells, a human cervical carcinoma cell line. Neither the R1 mRNA nor protein levels changed significantly when cells progressed from G0-G1 into early S phase and then into late S. In contrast, both R2 mRNA and protein levels were elevated as Caski cells entered S phase. As shown in our analysis of the R2 protein by two-parameter flow cytometry (Fig. 3), R2 protein levels reached their highest level in late S phase and then decreased as cells entered G2-M. Our data in this human cervical carcinoma cell line are in partial agreement with other studies using S49 mouse T lymphoma cells, bovine kidney MDBK cells, and mouse mammary tumor cells. The levels of R1 and R2 mRNAs, as well as R2 protein levels, were cell cycle dependent, whereas the R1 protein level was constant throughout the cell cycle (8–10). Our data are also in partial agreement with the results from leukemia L1210 cells, Ehrlich tumor cells in mice, and regenerating normal hepatocytes in rats, where both the R1 and R2 protein levels increased when cells entered S phase (13, 14). These various studies, including our data in Caski cells, suggest that activation of RR activity at the G1-S boundary is controlled by an up-regulation of either the R1 or R2 protein level, dependent on which RR subunit protein is the limiting factor for holoenzyme activity in a specific cell. Additionally, the reported variation in the expression of RR mRNA or protein levels throughout the cell cycle from these studies using different types of mammalian cells suggests that R1 and R2 mRNA and proteins must be regulated by multiple mechanisms for stability and biosynthesis throughout the cell cycle.

In addition to characterizing the cell cycle regulation of RR in Caski cells, another major aim of this study was to test whether IR had an effect on RR induction in these human cancer cells, as reported for other types of DNA-damaging agents (4-NQO, MMS, and UV light) in E. coli and yeast or mouse cells (15–25). A single dose of IR (6 Gy) was chosen, because this dose was used to study the HU-dependent radiosensitization in Caski cells, as described in a recent report from our laboratory (28). It is also in the range of doses used to detect IR-inducible genes, including thymidine kinase (another enzyme involved in dNTP biosynthesis) in other human cancer cell lines (44).

To exclude a cell cycle redistribution effect, a synchronized G0-G1 population was used to test the induction of RR by IR. These cells were maintained at confluence and in low serum for up to one cell cycle (30 h) after IR. A control for this experiment was a similar synchronized G0-G1 cell population, which was then replated at a low cell density in complete medium (10% FBS) without prior IR. The steady-state R1 protein level in a synchronized G0-G1 Caski cell population was not affected by IR nor serum stimulation, although a 2-fold increase in the mRNA level was found. In contrast, the R2 protein level showed a progressive increase after 6 Gy, resulting in a 17-fold increase within 18–24 h after IR, although these growth-arrested Caski cells were maintained at confluence and in low serum. Only a 2-fold increase in the level of R2 mRNA was found. However, if growth-arrested Caski cells were stimulated to proliferate by replating in complete medium (10% FBS) at low density without IR, then similar increased levels of R2 protein (18-fold increase) were found after 24–30 h, but there was an earlier >5-fold increase in R2 mRNA levels. Thus, although both growth stimulation by complete medium containing 10% FBS at low density and 6 Gy in low serum containing medium under contact-inhibited tissue culture conditions induced the same level of R2 protein, the R2 mRNA level in cells treated with IR was considerably lower than that in cells released into complete medium containing 10% FBS. These results suggest that the posttranscriptional regulation of the R2 subunit could be enhanced by IR, independent of cell cycle progression into S phase. Interestingly, the E2F1 nuclear protein level appears to be unrelated to posttranscriptional activation of R2 protein after IR in these human tumor cells.

The most reasonable hypothesis for up-regulation of the R2 subunit after IR (or other types of DNA damage) is that induction results in an increase of RR activity and consequently accelerates the production of dNTPs to facilitate efficient DNA repair synthesis. Using a CDP reductase in situ assay, RR activity was determined by the quantification of radiolabeled cytidine converted into dCTP and incorporated into genomic DNA. Our data showed that incorporation of radiolabeled cytidine into DNA was enhanced 4-fold by 6 Gy. These data

![Fig. 9. Western blot analysis of R1, R2, and E2F1 protein levels after replating of confluent and growth-arrested Caski cells in complete medium (10% FBS) without IR. Confluent and growth-arrested Caski cells were trypsinized and plated in 10% FBS containing medium at low density (5 × 10^6 cells/150-mm dish). Total protein extract (25 μg for R1 and R2) or nuclear extract (20 μg for E2F1) from the cells at each time point was loaded and fractionated on 10% SDS-PAGE. Proteins were then transferred to PVDF membranes for Western blotting. A human R1 monoclonal antibody, human R2 polyclonal antibodies, and a human E2F1 monoclonal antibody were used. A, an image of Western blots from a FluorImager. B, relative levels of R1, R2, and E2F1. Amount of R1, R2, and E2F1 at each time point was quantified by a FluorImager and normalized by the levels at 0 h as described in Fig. 2. •¿, R1; III, R2; ▲, E2F1.](image-url)

![Fig. 10. Cell cycle distribution after replating confluent and growth-arrested Caski cells in complete medium (10% FBS) without IR. Confluent and growth-arrested Caski cells were trypsinized and plated in complete medium (10% FBS) at low density (5 × 10^6 cells/150-mm dish). At each time point, cells were labeled with 20 μM of BrdUrd and fixed. After staining with a BrdUrd-monoclonal antibody, an anti-mouse-IgG-FITC conjugate, and PI, the cell cycle distribution was analyzed by flow cytometry. The percentage of cells in each cell cycle stage was analyzed using Lysis II software. The data were plotted as the means from three experiments; bars, SE. •¿, G1; III, early S; ▲, late S; ▼, G2-M; •, total S.](image-url)
also suggest that the R2 protein level may be a limiting factor for RR activity in G2-M synchronized Caski cells, because enhanced RR activity after IR was directly related to an up-regulation of the R2 protein levels, whereas no significant change of the R1 steady-state protein levels were observed.

It is important to point out that the activation of posttranscriptional regulation of R2 after IR in Caski cells appears to be different from the studies using other types of DNA-damaging agents, such as MMS, 4-NQO, UV light, HU, and chlorambucil in E.coli and yeast and mouse cells (15–27). These studies reported activated transcriptional expression of RR after these DNA-damaging agents, whereas no posttranscriptional response was found (or investigated). The effect of IR on the stability and/or the translation of R2 protein needs to be studied in Caski cells to further clarify how IR increases R2 protein level by a posttranscriptional mechanism. Although the molecular mechanisms for human R2 protein translation and stability have not been reported to date, translational control of the R2 subunit has been studied in some invertebrate organisms. It was found that maternal R2 RNA was not translated efficiently until within minutes of fertilization in clam and sea urchin oocytes (48). These data suggest that the marked increase in R2 subunit levels found in early development in these invertebrates was finely controlled by posttranscriptional regulation. Additionally, it was reported recently that a protein (p82) was bound to the 3′ untranslated region of R2 mRNA and that the subsequent phosphorylation of this p82 protein after fertilization in these oocytes was involved in the activated translation from masked R2 RNA (49). An in vitro kinase assay suggested that the cdc2 kinase inhibitor, p27KIP, prevented the phosphorylation of p82 and the translation of R2 in these oocytes (49).

In this study, we also found that IR stimulated confluent and growth-arrested Caski cells to enter S phase after IR under low-serum conditions. A similar observation was recently reported by Hallahan et al. (45) in three different human cell lines. As we discussed in a recent publication from our laboratory, abrogation of p53 and Rb functions in G1 checkpoint integrity dependent.

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