Cell division cycle 25 homolog c effects on low-dose hyper-radiosensitivity and induced radioresistance at elevated dosage in A549 cells

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The underlying mechanisms behind both low-dose hyper-radiosensitivity (HRS) and induced radioresistance (IRR), generally occurring at elevated radiation levels, remain unclear; however, elucidation of the relationship between cell cycle division 25 homolog c (Cdc25c) phosphatase and HRS/IRR may provide important insights into this process. Two cell lines with disparate HRS status, A549 and SiHa cells, were selected as cell models for comparison of dose-dependent Cdc25c phosphatase expression subsequent to low-dose irradiation. Knockdown of Cdc25c in A549 cells was mediated by transfection with a pGCsi-RAN-U6neo vector containing hairpin siRNA sequences. S216-phosphorylated Cdc25c protein [p-Cdc25c (Ser216)], cell survival and mitotic ratio were measured by western blot, colony-forming assay and histone H3 phosphorylation analysis. Variant p-Cdc25c (Ser216) expression was observed in the two cell lines after irradiation. The p-Cdc25c (Ser216) expression noted in SiHa cells after administration of 0–1 Gy radiation was similar to the radioresistance model; however, in A549 cells, the dose response for the phosphorylation of the Cdc25c Ser216 residue overlapped the level required to overcome the HRS response. Furthermore, Cdc25c repression prior to low-dose radiation induced more distinct HRS and prevented the development of IRR. The dose required to overcome the HRS response coincided with the effect of early G2-phase checkpoint arrest in A549 cells (approximately 0.3 Gy), and Cdc25c knockdown in A549 cells (approximately 0.5 Gy) corresponded to the phosphorylation of the Cdc25c Ser216 residue. Resultant data confirmed that dose-dependent Cdc25c phosphatase does effectively act as an early G2-phase checkpoint, thus indicating mechanistic importance in the HRS to IRR transition in A549 cells.

Keywords: low-dose irradiation; HRS/IRR; Cdc25c; cell cycle arrest

INTRODUCTION

Radiation therapy, often coupled with surgery and chemotherapy, is also one of the most important methods of contemporary cancer treatment [1]. More than 60% of all cancer patients will require radiotherapy at various stages over the course of their treatment. Though the technology for clinical administration of radiation therapy and radiation dose fractionation has greatly improved over the past decades, increasing application of these techniques warrants increasing concern as to the effects of low-dose radiation [2–5]. In addition to the previously considered roles of adaptive responses, bystander effects and genetic instability, the phenomenon of low-dose hyper-radiosensitivity (HRS) is one of the important biological effects of low-dose ionizing radiation, and HRS may play an as yet undefined role in the outcomes of cancer treatment [6–7]. It has been confirmed both in vitro and in vivo that numerous tumor types as well as several normal tissues show hypersensitive responses at very low radiation doses; however, the same tumors and tissues exhibit induced radioresistance (IRR) at elevated radiation levels [8]. Because of the important biological consequences of the HRS/IRR phenomenon, it is considered to be of clinical and therapeutic significance, playing a particularly notable role in the treatment of refractory cancers with conventional radiotherapy and reirradiation treatments.
Explanation of the mechanism of low-dose HRS has been recently attempted by several research groups, though IRR is rarely considered simultaneously. These studies indicate that dose-dependent low-dose irradiation induces early checkpoint arrest at the G2/M phase of the cell cycle, indicating the likely predominant mechanism of both HRS and IRR [9]. Krueger et al. found that the dose-activation profiles for both ataxia-telangiectasia mutated (ATM) activity and the early occurrence of the G2/M checkpoint correspond to the transition in cell survival from low-dose HRS to IRR [10]. Furthermore, abduction of the checkpoint by inhibition of checkpoint kinase 1 (Chk1) and checkpoint kinase 2 (Chk2) has also increased low-dose radiosensitivity [11]. These results suggest that the ATM-Chk1/Chk2 checkpoint pathway is important in the transition from HRS to IRR. However, at the molecular level, multiple kinases contribute to the arrest of early G2/M checkpoints to ensure that the cell is prepared for normal mitosis [12, 13]. At present, the molecular mechanisms involved in many of the early G2/M checkpoints in HRS/IRR are still poorly defined.

DNA double-strand breaks (DSBs) are biologically significant lesions induced by low-dose ionizing radiation [14]. In response to DSBs, the ATM-Chk1/Chk2 checkpoint pathway is activated to prevent cell cycle progression. The mitotic inducer, Cdc25c phosphatase, is a downstream component of the ATM-Chk1/Chk2 checkpoint pathway that is activated by ionizing radiation in response to DSBs [15]. Phosphorylation of Serine 216 residues (S216) by Chk1 and Chk2 has been shown to inhibit Cdc25c activation, thus arresting cells in G2/M phase [16, 17]. Therefore, it was hypothesized that Cdc25c phosphatase was likely involved in the underlying control mechanisms behind the shift in cell behavior between HRS and IRR.

The role of Cdc25c phosphatase in the shifting cell behavior between HRS and IRR was examined in the current study using two distinct tumor cell lines of disparate HRS status. Cell lines A549 and SiHa, the two most commonly examined tumor cell lines in radiobiological research, were selected as models. The dose-dependent expression of Cdc25c protein and S216-phosphorylated Cdc25c protein [p-Cdc25c(Ser216)] were analyzed after low-dose irradiation of both cell lines. Moreover, the effects of Cdc25c down-regulation in A549 cells on HRS/IRR and G2/M arrest were examined. This study provides unique insight into the precise molecular mechanism underlying HRS/IRR.

**MATERIALS AND METHODS**

**Cell culture**

Human lung adenocarcinoma bronchioloalveolar carcinoma A549 and human cervical cancer SiHa cell lines were kindly provided by the Gastrointestinal Surgery Institute of Union Hospital in Tongji Medical College of Huazhong University of Science and Technology (Wuhan, China). Cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal bovine serum (GIBCO BRL, Eggenstein, Germany), 3 mmol/l glutamate (Gibco BRL), 100 U/ml penicillin and 100 µg/ml streptomycin (Gibco BRL) under standard incubator conditions (37°C, 5% CO2).

**Irradiation**

For cell survival assays, cells were irradiated using a Siemens Primus K X-ray unit (Primus, Siemens AG, Erlangen, Germany) with single doses administered at a dose rate of 0.8 Gy min−1. The cells were irradiated within a dose range of 0.1–2.0 Gy and then harvested at different times for cell extracts.

**Generation of the Cdc25c shRNA plasmids**

The small hairpin RNAs (shRNAs) targeting Cdc25c were commercially designed and synthesized by Genechem Co. Ltd (Shanghai, China). The shRNA expression cassette consisted of a 21-bp target gene specific sequence, a 9-bp loop and another 21-bp reverse complementary sequence, all influenced by a human U6 promoter. A termination sequence (TTTTT) was located immediately downstream of the second 21-bp reverse complementary sequence to terminate transcription by RNA Pol III. Target DNA fragments to the coding region were approximately 1907 bp and 774 bp and 970–990 bp, respectively. Enzyme restriction sites of BamHI and HindIII were constructed into the extreme of the oligonucleotide fragments. The specificity of the constructed oligonucleotide fragments was analyzed by BLAST to ensure a match to the target gene. The sequences used were as follows:

| shRNA1 | 5′-GGATCCGGACAACAATACCAGATAATTC AGAGATTTATCTCTGTTGGATCGTTTTGGATAGCT- T-3′; shRNA2, 5′-GGATCCGTTCCAATACTACTGTTCGTTCTCAAGAGGATCTATGGAATATGTAAGATTAATTTTGGAGCTGAG -TAGCTT-3′; and shRNA3, 5′-GGATCCCCATCTCTCTGCACTCAAGGCTTCAAGAGGCTTGAAGGTGATAGCATATGATATTTTGGATAGCTT-3′. |
|---|---|

A nonsense sequence shRNA was constructed as a positive control as follows: 5′-GGATCCCTTCTCCGAACTGTGTC ACGTTTCTCAAGAGGATCGAGACGCTCTTCGGAGATAATTTTGGAATAGCTT-3′. After purification, the shRNA oligonucleotide fragments were ligated into the pGCsi-U6/Neo/DsRed vector (Genechem Co. Ltd, Shanghai, China). The Cdc25c shRNA recombinants were identified by enzyme digestion and sequencing (Genechem Co., Ltd, Shanghai, China). The red fluorescent colonies were selected after 2 weeks and then maintained in 600 µg/ml G418. Reverse transcription polymerase chain reaction (RT-PCR) and
western blot were applied to analyze Cdc25c mRNA and protein levels, respectively.

**Clonogenic cell survival assay**
Immediately following irradiation, cells were trypsinized and diluted in phosphate-buffered saline (PBS). Flow cytometry (FACS Vantage, BD Bioscience, San Jose, CA, USA) was used to precisely quantify cell numbers. Cells were plated at 200 cells per 60-mm culture dish. Three replicates were prepared for each data point. After 10–14 days of incubation, the cells were fixed in 95% methanol and stained with crystal violet (100% methanol solution). A population of >50 cells were counted as one survived colony. Assays were repeated three times.

Dose–survival curves were generated from the average survival levels of the three data points. The data were fitted to a modification of the linear-quadratic (LQ) model called the induced-repair (IR) model using non-linear least-squares regression. Survival fraction, measured at the tested doses, was fitted with the SAS JMP software (SAS Institute, Cary, NC, USA) to produce the best-fit parameters. The IR model is described as [18]:

\[
SF = \exp\left(-\alpha_r[1 + (\alpha_r/\alpha_i - 1) \exp(-D/D_c)]D - \beta D^2\right)
\]

Where \(\alpha_r\) is the low-dose value of \(\alpha\) (derived from the response at very low doses), \(\alpha_i\) is the value extrapolated from the conventional high-dose response, \(D\) is the radiation dose, \(\beta\) is a constant (as in the linear-quadratic equation) and \(SF\) is the fraction of cells surviving at dose \(D\). The presence of low-dose HRS was deduced from the values of \(\alpha_r\) and \(\alpha_i\) without overlap of confidence limits and from a \(D_c\) value (dose transition between hyper-radiosensitivity and increased resistance) significantly greater than zero.

**RT-PCR**
Total RNA was extracted from A549 and SiHa cells using Trizol reagent (Life Technologies Inc., Gaithersburg, MD, USA) according to the protocol provided by the manufacturer. In brief, cDNA was synthesized from 2 µg of total RNA using superscript reverse transcriptase (Life Technologies Inc.). The glyceraldehyde 3-phosphate dehydrogenase (GAPDH) gene was used as an internal control. Primer sequences for Cdc25c detection were as follows: 5’-TTGTAGCAGTCCGAATGGTT-3’ (forward) and 5’- CTCTGCTGCTGCTTCCTCTCCT-3’ (reverse). The amplification conditions were 94°C for 10 min followed by 30 cycles of 94°C for 30 s, 56.6°C for 30 s and 72°C for 30 s with an additional 10 min 72°C extension. PCR products were analyzed by 1.2% agarose gel electrophoresis with ethidium bromide using UV light transilluminator visualization.

**Western blot analysis**
Polyclonal antibodies against Cdc25c and phosphorylated Cdc25c at Ser216 were purchased from Cell Signaling Technology (Danvers, MA, USA). Confluent cells were lysed in lysis buffer (20 mM Tris-HCl, pH 7.5, 150 mM sodium chloride, 1 mM EDTA, 1 mM EGTA, 1% Triton, 2.5 mM sodium pyrophosphate, 1 mM h-glycerolphosphate, 1 mM sodium orthovanadate, 0.5 mM phenylmethylsulfonyl fluoride and 1 mg/ml leupeptin). Protein lysates (200 µg/lane) were subjected to 8% polyacrylamide gel electrophoresis and transferred onto a nitrocellulose membrane. Non-specific binding sites were blocked by incubating the nitrocellulose membrane for 1 h at 37°C with 5% nonfat dried milk in Tris-buffered saline containing 0.05% Tween-20 (TBST). Membranes were incubated overnight at 4°C with primary antibodies (1:500 dilution). Then, membranes were additionally washed and incubated with horseradish peroxidase-conjugated secondary antibodies (1:1000 dilution). Bands were visualized using an enhanced chemiluminescence system (ECL, Pierce, Rockford, IL, USA).

**Assessment of phosphorylated histone H3 by flow cytometry**
Cells in the exponential phase of growth were irradiated at different doses (0 Gy, 0.3 Gy and 0.5 Gy) at 37°C and harvested after radiation exposure. These cells were then trypsinized, fixed in 2% paraformaldehyde, permeabilized with 1% Triton X-100 (Sigma) and stained with anti-phospho-histone 3 (Ser 10)-FITC (Cell Signaling Technology, Danvers, MA, USA) in a 1:2000 dilution at 4°C with primary antibodies (1:500 dilution). Then, membranes were additionally washed and incubated with horse-radish peroxidase-conjugated secondary antibodies (Becton Dickinson, Mississauga, CA, USA). The mitotic ratio was determined as the ratio of irradiated to unirradiated cells staining positive for phosphorylated histone H3 in matched cell cultures.

**Statistical analysis**
All values in the text and figures are expressed as the mean ± SEM. Statistical significance was determined by a Student’s t-test using the software package SPSS (http://SPSS.com, Chicago, IL, USA) for Windows 11.5. A value of \(P < 0.05\) was considered statistically significant.

**RESULTS**

**HRS/IRR and Cdc25c activity in A549 and SiHa cells**
Previous studies indicated that the ATM-Chk1/Chk2 checkpoint pathway is important in the cellular transition from HRS to IRR; however, the effector checkpoint kinase Cdc25c located downstream from these proteins has never
been studied in conjunction with HRS. The involvement of Cdc25c in HRS/IRR is, however, explored over the course of the current study. First, the low-dose cell survival of A549 and SiHa cells was measured. Survival of SiHa cells was decreased in a dose-dependent manner, and no evidence of HRS/IRR was exhibited (Fig. 1A). In A549 cells subjected to X-ray doses >0.3 Gy, the dose–survival relationship conformed to the conventional LQ model. When extrapolated backward below 0.3 Gy, a hypersensitive response to X-rays was noted (Fig. 1). The $D_c$ value, marking the transition point in dosage at which cells were observed to switch between HRS and IRR, was 0.27 Gy ($\pm 0.08$) (Table 1).

Subsequently, p-Cdc25c(Ser216) and Cdc25c expression following low-dose X-ray irradiation of A549 and SiHa cells were measured by western blot analysis. A different p-Cdc25c(Ser216) expression was observed in the two cell lines after irradiation. In SiHa cells that did not exhibit the HRS/IRR phenomenon, a similar p-Cdc25c(Ser216) level occurred with irradiation of 0–1 Gy (Fig. 1C, E). In A549 cells, however, much lower p-Cdc25c(Ser216) levels were noted at <0.3 Gy, indicating a possible insufficient phosphorylation of the Ser216 of Cdc25c. An increase in phosphorylation at radiation levels between 0.3–0.5 Gy was subsequently observed, with little further increase as the dose exceeded 0.5 Gy (Fig. 1D, F). The dose required to

**Figure 1:** Low-dose clonogenic survival and expression levels of cell division cycle 25C phosphatases in SiHa and A549 cells (A, B) Clonogenic survival of SiHa cells (A) and A549 cells (B) by X-ray irradiation dose. HRS is observed in A549 cells but not in SiHa cells. (C, D) Cdc25c and p-Cdc25c(Ser216) expression by X-ray irradiation dose in SiHa cells (C) and A549 cells (D). (E, F) The levels of Cdc25c and p-Cdc25c(Ser216) expression observed in SiHa cells and A549 cells were quantified by band intensities and normalized to that of $\beta$-actin (mean $\pm$ standard error; $n = 3$), showing dose-dependent Cdc25c Ser216 phosphorylation from 0 Gy to 0.3 Gy in A549 cells but not in SiHa cells.
overcome the HRS response (approximately 0.3 Gy) corresponded to the dose necessary for the phosphorylation of Ser216 in Cdc25c in A549 cells. These data established that the pattern of p-Cdc25c (Ser216) expression corresponds with the transition from HRS to IRR in A549 cells.

Knockdown of Cdc25c expression by Cdc25c shRNAs
A549 cells were individually transfected with three different Cdc25c-shRNAs and an empty plasmid pGCsi, and then the cells were stably screened by 600 µg/ml G418. A549 clones stably expressing Cdc25c-shRNAs were selected, and Cdc25c expression was further identified by RT-PCR and western blot analyses. As shown in Fig. 2A and C, the transfection of A549 cells with Cdc25c-shRNA2 and Cdc25c-shRNA3 resulted in a significant decrease in Cdc25c mRNA expression. The amount of Cdc25c in A549 cells transfected with Cdc25c-shRNA2 and Cdc25c-shRNA3 decreased by 66% and 74%, respectively, compared with the amounts observed in the pGCsi (control shRNA) and untreated A549 cells. No significant difference was observed in the mRNA expression level between A549 cells treated with Cdc25c-shRNA1, control shRNA and untreated A549 cells. As shown in Fig. 2B and D, a similar trend was observed in Cdc25c protein expression by western blot analyses. The figure showed a 65% and 75% reduction by Cdc25c-shRNA2 and Cdc25c-shRNA3, respectively, without any significant modulation of the amount of β-actin. Therefore, Cdc25c-shRNA3 (targeting approximately 970–990 bp) was selected to inhibit Cdc25c expression because of the relatively high inhibitory effectivity.

Measurement of low-dose HRS and Cdc25c phosphatase after Cdc25c knockdown in A549
In order to investigate the existence of HRS/IRR in A549 cells and the involvement of Cdc25c, clonogenic cell survival assays were performed in pGCsi-A549 cells and Cdc25c-shRNA-A549 cells. Compared with pGCsi-A549 cells, the knockdown of Cdc25c prior to low-dose radiation induced a more distinct HRS/IRR and prevented the development of IRR (Fig. 3A, B). The expression of p-Cdc25c (Ser216) after irradiation with variant X-ray doses was found to be much weaker in Cdc25c-shRNA3-A549 cells.
than in A549 and pGCsi-A549 cells (Fig. 3C, D, E and F). In Cdc25c-shRNA3-A549 cells, an obvious increase in p-Cdc25c (Ser216) expression was observed from 0.5 to 1 Gy of radiation compared with the levels observed below 0.5 Gy (Fig. 3D and F). Dose-dependent p-Cdc25c (Ser216) expression was found in both A549 cells and pGCsi-A549 control cells. The $D_0$ value was 0.30 Gy (±0.05) in pGCsi-A549 cells and 0.51 Gy (±0.13) in Cdc25c-shRNA3-A549 cells (Table 1).

The Cdc25c knockdown can be deduced to be the molecule that reduces p-Cdc25c (Ser216) levels in A549 cells, as seen in Fig. 3. The dose required to overcome the HRS response (approximately 0.5 Gy) closely corresponded to the dose necessary for Cdc25c phosphorylation in Cdc25c knockdown A549 cells. The data establish that the pattern of Cdc25c phosphorylation corresponds with the transition from HRS to IRR.

**Measurement of low-dose radiation-induced early G2/M checkpoint response after Cdc25c knockdown**

The effect of the irradiation-induced function of the early G2 checkpoint in A549 cells, pGCsi-A549 control cells and Cdc25c-shRNA-A549 cells was assessed by the
progression of irradiated G2 cells into mitosis through the use of a mitosis-specific marker, phosphorylated histone H3. The observed decrease in the mitotic ratio indicated that radiation-induced G2-phase cells were arrested in mitosis. The baseline mitosis ratios of the three cell lines were similar, as shown in Fig. 4A. G2 arrest was evident up to 30 min after exposure to 0.3-Gy radiation in A549 cells and control cells, but not in Cdc25c knockdown A549 cells (Fig. 4B). These data indicate that the early G2 checkpoint plays a key role in Cdc25c knockdown A549 cells at doses of 0.3 Gy. G2 arrest occurred, however, after exposure to 0.5-Gy radiation in both A549 cells and Cdc25c knockdown A549 cells (Fig. 4C). Based on these findings, the radiation dose at the early G2 phase checkpoint arrest is coincident with the dose necessary to overcome the HRS response and Cdc25c phosphorylation.

DISCUSSION

The novel radiobiological phenomenon of HRS/IRR, an effect in which cell death is induced by excessive sensitivity to small single doses of ionizing radiation but resistance (per unit dose) to larger single doses increases, has been observed in cell lines of lung adenocarcinoma, colorectal carcinoma, bladder carcinoma, melanoma, prostate carcinoma, cervical squamous carcinoma, neuroblastoma and glioma, as well as in a single nonmalignant lung epithelial cell line and a single primary human fibroblast cell line [6]. Though HRS/IRR is a widely occurring phenomenon, the effect has been shown to be more prominent in proliferating malignant tissues than in quiescent normal tissues [19, 20]. In addition, tumorigenic cells with high metastatic potential have been demonstrated to preferentially exhibit HRS at a much greater level than that seen in poorly metastatic cells.
This observation initially prompted exploration of the Cdc25c phosphatase in HRS/IRR in A549 cells. The knockdown of Cdc25c by shRNA subsequently resulted in repression of p-Cdc25c (Ser216) expression as shown in Fig. 3C and D, as well as in the trend of the p-Cdc25c (Ser216) expression level in Cdc25c-shRNA-A549 cells. This indicated that Cdc25c still coincided with the transition in the survival response from HRS to IRR (Fig. 3B, D and F). Furthermore, knockdown of Cdc25c induced a significantly more distinct HRS and prevented the development of IRR (Fig. 3B). The dose points at which G2 arrest and Cdc25c knockdown occurred in A549 cells were coincident with the doses needed to overcome the HRS response, suggesting a correlation (Fig. 4). Cdc25c serves as a factor controlling mitotic initiation, providing a link between overcoming HRS and G2 arrest in A549 cells. Thus, the dose-dependent Cdc25c phosphatase is an important regulator of the HRS/IRR transition.

In previous studies concerning HRS/IRR, a distinct pattern in ATM phosphorylation has been consistently observed [10, 11]. Although radiation-induced ATM Ser1981 phosphorylation has been shown to be the key determinant for overcoming HRS and establishing the IRR survival response, it is not the only factor involved in the transition. The present study demonstrates that a similar phosphorylation pattern of Cdc25c exists in HRS/IRR as in ATM, consistent with previous reports. Thus, it is likely that a newly described cell cycle checkpoint p-Cdc25c (Ser216) is also important in the transition from HRS to IRR in A549 cells. In light of the ATM-Chk1/Chk2-Cdc25c pathway’s general activation by irradiation-induced DNA damage [28], activation of this pathway may additionally be involved in the HRS/IRR transition. Further research will be required to clarify the role of each protein involved in the ATM-Chk1/Chk2-Cdc25c pathway associated with HRS/IRR.

Non-small cell lung cancer (NSCLC) is among the most common malignancies in the world, and radiotherapy is one of the most effective nonsurgical treatments for patients suffering with this disease [30]. The potential for hyperfractionation to improve the therapeutic ratio of radiation treatments for NSCLC was recognized through failure of large-dose fractionation to improve local control and the observation that smaller fractions were associated with fewer effects in normal tissues [31]. Understanding the mechanism of HRS/IRR in lung cancer cells can aid in the optimization of the therapeutic ratio, allowing successful treatment with smaller dose fractions. The current findings suggest that Cdc25c may be a potentially useful target when multiple small-dose fractions of radiation are employed for the development of more effective radiation strategies. The first robust evidence for the role of Cdc25c phosphatase in HRS/IRR in A549 cell lines is contained in the current study, suggesting that Cdc25c may act as a
promising new target for the development of effective radiation for NSCLC by hyperfractionation.

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