Overexpression of CDC25B Overrides Radiation-induced G2-M Arrest and Results in Increased Apoptosis in Esophageal Cancer Cells

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ABSTRACT

CDC25B phosphatase plays a key role in controlling G2-M progression by dephosphorylating two inhibitory residues of CDC2 and also has been suggested to have an oncogenic property. In this study, we investigated the effect of CDC25B overexpression on radiation-induced G2-M arrest and radiation sensitivity in esophageal cancer cells. TE8-CDC25B, in which CDC25B was overexpressed under an inducible system, was more radiosensitive than the vector control (TE8-neo) in a clonogenic survival assay. Without radiation, CDC25B overexpression had little effect on cell cycle fractions or growth rate. After 10-Gy radiation, TE8-CDC25B showed decreased G2-M arrest and increased apoptosis, whereas TE8-neo displayed prolonged G2-M arrest and less apoptosis. During this period, there were no differences in the protein amounts of CDC2 and cyclin B1 between the two cell lines. However, more CDC25B expression, which was reduced immediately by radiation, was sustained in TE8-CDC25B than in TE8-neo. Moreover, induction of tyrosine phosphorylation of CDC2 and reduction of CDC2 kinase activity after irradiation was less significant in TE8-CDC25B than in TE8-neo. These results indicate that cancer cells that overexpress CDC25B override G2-M arrest by retaining CDC2 kinase activity and undergo apoptosis after radiation. This may point to an effective approach toward improving radiotherapy outcomes of various cancers.

INTRODUCTION

G2-M arrest and apoptosis are common phenomena after DNA-damaging treatment such as irradiation. In recent years, the mechanism of G2-M arrest after DNA damage has been elucidated as follows. When DNA is injured by irradiation, Chk1 and Chk2, which functions in an ATM-dependent manner (1, 2), are both activated (1–5). They then inactivate CDC25 by phosphorylating the serine-216 residue (1, 2, 4, 5). During the G2 phase, CDC2 forms a heterodimeric complex with cyclin B1 but remains inactive by phosphorylation of CDC2 on tyrosine-15 and threonine-14 residues (6, 7). CDC25 activates the CDC2/cyclin B1 complex by dephosphorylating these inhibitory residues (8–10), and this step is indispensable for the entry to mitosis. Eventually, DNA damage results in cell cycle arrest at G2-M transition through inactivation of CDC25.

On the other hand, DNA injuries can be restored by various systems during G2-M arrest. Interestingly, yeast experiments revealed that, although both the DNA repair system and G2-M arrest are required for adequate DNA restoration (11, 12), they are independently regulated by different genes. Thus, when the duration of G2-M arrest is not sufficient, cells with DNA injuries display immature mitosis or mutagenesis (13, 14). Furthermore, they would be eliminated as apoptotic cells if the DNA injury is lethal. Although it is not fully understood how DNA damage causes cells to undergo apoptosis at the G2-M checkpoint, this pathway is one of the most important mechanisms in the radiation therapy of malignant tumors. We have investigated the expression of G2-M regulating proteins in association with radiation sensitivity in esophageal cancer patients, and we found that overexpression of CDC25B in esophageal cancer cells is associated with a high sensitivity to radiotherapy (15).

In human cells, the CDC25 gene family includes three homologues: CDC25A, CDC25B, and CDC25C (16, 17). CDC25A is expressed in late G1 and is essential for G1-S phase transition, activating the cyclin E/Cdk2 complex (18, 19). Both CDC25B and CDC25C, which are 45% identical at the amino acid level (85% identical in the catalytic domains), are predominantly present in the G2 phase and are necessary for cells to enter mitosis (20–22). Among these three homologues, CDC25A and CDC25B are frequently overexpressed in human cancers and experimentally exhibit oncogenic potentiality. In rodent fibroblasts, both CDC25A and CDC25B can elicit transforming activity in cooperation with activated Ras and loss of Rb (23, 24). Furthermore, CDC25B has been reported to induce hyperplasia and increase susceptibility to carcinogen-induced tumors in transgenic mice (25, 26). The expression of CDC25A and CDC25B are regulated at the transcriptional level, such as by c-myc or transforming growth factor-β (27, 28). Finally, overexpression of CDC25A and CDC25B was observed in various human cancers, including those of the lung, stomach, head and neck, large intestine, esophagus, and lymph nodes, and were associated with poor prognosis (24, 29–32).

Radiation therapy has been successful in clinical treatment because cancer cells generally show higher sensitivity to radiation than do noncancerous tissues. We, therefore, hypothesized that CDC25B, as an oncogene, increases radiation sensitivity. In this study, we investigated the effect of CDC25B overexpression on cell cycle progression and apoptosis after radiation using TE8 esophageal cancer cells.

MATERIALS AND METHODS

Cell Culture and Induction of Overexpression of CDC25B. CDC25B2 plasmids (pGEM) containing MMTV-long terminal repeat upstream of human CDC25B2 (kindly provided by Dr. David Beach, Cold Spring Harbor Laboratory, Cold Spring Harbor, New York) and plasmids (pGEM) without CDC25B2 as a control were cotransfected with pcDNA3 into the TE8 esophageal cancer cell line by the lipofectin method. After 2 weeks, colonies resistant to G418 (Promega, Madison, WI) were collected and maintained in RPMI 1640 containing 10% fetal bovine serum. Growing cells were trypsinized and replaced at 10× dilution once a week. The expression of CDC25B was examined after 10 passages, and, thereafter, other experiments were performed until 20 passages. Cells were stimulated by dexamethasone at various concentrations for the indicated periods of time, and then cell extracts were collected and subjected to Western blotting to confirm the induction of CDC25B expression under MMTV promoter. In all of the experiments after that, the cells were incubated with 1.0 μM dexamethasone for more than 48 h to induce CDC25B expression. The clonal diversity of MMTV-CDC25B2 transfectant (TE8-CDC25B) was investigated by

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3 The abbreviations used are: ATM, ataxia telangiectasia-mutated; MMTV, mouse mammary tumor virus.

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subcloning it by the limiting dilution method. Five of 20 subclones did not show CDC25B induction by dexamethasone. The remaining 15 clones showed obviously higher expression of CDC25B in Western blotting, ranging from 2- to 18-fold that of the vector alone (TE8-neo) by densitometer.

Clonogenic Survival Assays. Four thousand cells were placed in a 10-cm dish, and, after 24 h, were exposed to various doses of radiation using a 137Cs irradiation unit with a dose rate of 1.2 Gy/min. After 14 days of incubation at 37°C, the cultures were fixed in 100% ethanol and stained with Giemsa solution (Sigma Chemical Company, St. Louis, MO). Colony-forming efficiency was calculated as the average of triplicate experiments by counting the number of colonies that consisted of >50 cells.

Determination of Cell Cycle and Apoptosis by Flow Cytometry. The 50% confluent cells, which were growing in the exponential phase, were exposed to 10 Gy of radiation. In the subsequent period, adherent cells were trypsinized and mixed with floating cells, then fixed in 70% ethanol, and stored at 4°C. Cell suspensions were washed with PBS and incubated with 0.2 mg/ml RNase for 30 min at 37°C; then double-strand DNA was stained with 0.2 mg/ml propidium iodide. Measurement of DNA content for 10,000 cells in each sample was performed on a FACScan (Becton Dickinson), and the DNA histograms were interpreted using ModFit software.

Western Blotting and Antibodies. After washing with ice-cold PBS, the cells were lysed on ice in a lysis buffer [50 mM HEPES (pH 7.4), 150 mM NaCl, 20 mM EDTA, 1.0 mM DTT, 0.1% Tween 20, 1% phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin, and 10 μg/ml leupeptin] and centrifuged at 12000 rpm at 4°C for 20 min. Supernatant proteins (50 μg) diluted with SDS sample buffer were separated by 10% polyacrylamide gel electrophoresis, followed by electroblotting onto a polyvinylidene difluoride membrane. After blocking in 5% nonfat dried milk in Tris buffered saline (TBS), the membrane was incubated with the following primary antibodies for 1 h: cyclin D1 (M-20; Santa Cruz Biotechnology, Santa Cruz, CA), cyclin B1 (H-433; Santa Cruz), CDC2 (Transduction Laboratories, Lexington, KT), CDC25B (C-20; Santa Cruz), CDC25C (C-20; Santa Cruz), phosphotyrosine (clone 4G10; Upstate Biotechnology, Lake Placid, NY), and E-cadherin (HECD1; Takara Shuzo, Shiga, Japan) The protein bands were detected using the manufacturer’s instructions.

For detection of tyrosine phosphorylation on CDC2, 500 μg of cell lysates were obtained with the lysis buffer containing 0.5 mM sodium orthovanadate. The sample was precleared with protein A-Sepharose, then incubated with 2 μg of anti-CDC2 antibody and 30 μl of protein A-Sepharose. The immunocomplex was extensively washed with PBS, solubilized with SDS sample buffer, and subjected to Western blotting with antiphosphotyrosine antibody.

Cell Synchronization and CDC2 Kinase Assay. Cells arrested in the S phase were obtained by the double thymidine block method as described previ-ously (14). Four h after release from the thymidine block, the synchronized cells were just before the G2 phase and were exposed to 10 Gy of radiation. At subsequent time points, CDC2 kinase assay was performed as described previously (33). In this experiment, the cells were lysed on ice with lysis buffer, containing 10 mM β-glycerophosphate, 1 mM NaF, and 0.1 mM sodium orthovanadate. After centrifugation, the clarified supernatant material was preincubated with incubation protein A-Sepharose beads for 1 h at 4°C and incubated with CDC2 antibody for 1 h at 4°C, followed by incubation with antiimmunoglobulin IgG goat IgG for 1 h at 4°C. The immunocomplex was recovered with protein A-Sepharose and washed three times with lysis buffer and washed two times with reaction buffer [50 mM HEPES (pH 7.4), 10 mM MgCl2, 1 mM DTT, 2.5 mM EGTA, 10 mM β-glycerophosphate, 1 mM NaF, and 0.1 mM sodium orthovanadate]. The CDC2 immunoprecipitates were incubated with 1 μg of histone H1 (Boeringer Mannheim, Mannheim, Germany) and 5 μCi of [γ-32P]ATP in 50 μl of reaction buffer, for 15 min at 30°C. The reaction was terminated by adding SDS sample buffer, and the mixture was loaded onto a 10% SDS-polyacrylamide gel after boiling at 95°C for 5 min, and then the gel was autoradiographed.

RESULTS

Establishment of TE8 Overexpressing CDC25B. To investigate the effect of CDC25B overexpression on radiosensitivity, we used TE8 esophageal cancer cells transfected with a CDC25B expression vector under MMTV promoter (TE8-CDC25B) and with vector alone (TE8-neo). The induction of CDC25B protein in TE8-CDC25B was strongest, and the difference against vector control was most significant at 48 h after dexamethasone treatment at more than 1.0 μM (Fig. 1, a and b). The expression of CDC25B mRNA, which was confirmed by reverse transcription-PCR, was much stronger in TE8-CDC25B than in the vector control. There was no difference in cell growth, morphology, and spontaneous cell death between TE8-CDC25B and TE8-neo after dexamethasone treatment as well as in normal media without dexamethasone.

Clonogenic Survival Assay after Radiation. To assess the radi-ation sensitivity, a standard clonogenic survival assay was performed. Before radiation treatment, there was no difference in plating efficacy: 25.4% in TE8-CDC25B and 28.2% in TE8-neo. After radiation treat-ment with a dose of 2–8 Gy, TE8-CDC25B showed higher radiation sensitivity, forming fewer colonies than the vector control (Fig. 2). When dexamethasone was removed, TE8-CDC25B showed higher colony formation efficacy, whereas TE8-neo did not change. As a result, there was no difference in the radiation sensitivity of the two cell types without dexamethasone.

Cell Cycle Progression and Apoptosis after Radiation. Without radiation, there was no significant difference in the proportion of each
cell cycle phase between TE8-CDC25B and TE8-neo, growing in the exponential and asynchronous status, and the number of cells in spontaneous apoptosis was negligible in both cells. After 10-Gy radiation treatment, G1 arrest did not occur, but G2-M arrest was significant, reaching a maximum at 24 h postirradiation in both types of cells. A slight increase of the S-phase fraction was observed, but no difference was found between the two types of cells. TE8-CDC25B had fewer cells in G2-M than did TE8-neo at 24 h (33.1 versus 54.0%) or 36 h (13.1 versus 28.8%; Table 1). At 48 h postirradiation, TE8-CDC25B recovered to almost the normal cell cycle, whereas a relatively high proportion of the vector control (14.7%) was still in the G2-M phase. Thus, TE8-CDC25B showed less and shorter G2-M arrest after radiation than did TE8-neo. Apoptotic cells were detected in the sub-G1 fraction in the DNA histogram and were also confirmed under fluorescent microscope as nuclear condensation. Radiation-induced apoptosis was not significant until 12 h, when G2-M arrest was already observed in both cells, and reached maximum at 36 h postradiation, when most of the cells showed recovery from G2-M arrest. No further increase of apoptotic cells was observed after 48 h. There was a big difference in the proportion of apoptotic cells between TE8-CDC25B and TE8-neo. It was notable that TE8-CDC25B displayed more apoptotic cells than TE8-neo after exposure to 10-Gy radiation, because the proportion of apoptosis in each cell line was 32.3 versus 18.7% at 24 h, and 56.7 versus 34.8% at 36 h (Fig. 3). Without dexamethazone, G2-M arrest after radiation was slightly prolonged; however, there was no difference in the proportion of each cell cycle phase and the amount of apoptosis between the two cell lines (Fig. 3).

Effect of CDC25B Overexpression on Expression of Regulators of Cell Cycle after Radiation. To examine the mechanism for abrogation of G2-M arrest and increased apoptosis by CDC25B overexpression, expression of CDC25B as well as of other cell cycle-

<table>
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<tr>
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<th>0 h</th>
<th>12 h</th>
<th>24 h</th>
<th>36 h</th>
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<td>50.9 (45.7)</td>
<td>36.3 (29.5)</td>
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<td>29.5 (26.5)</td>
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<td>19.6 (17.6)</td>
<td>54.0 (43.9)</td>
<td>28.8 (18.8)</td>
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<tr>
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<td>(10.2)</td>
<td>(18.7)</td>
<td>(34.8)</td>
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<td>(100)</td>
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<td>53.4 (36.2)</td>
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Numbers in parenthses are percentages.
regulating molecules, including cyclin D1, cyclin B1 CDC25C, and CDC2, were investigated after radiation. Expression of cyclin D1 and CDC2 protein did not change after 10 Gy of radiation treatment in both cell lines (Fig. 4a). Cyclin B1, which is generally detected in the G2 phase and the early mitotic phase during the cell cycle, was strongly expressed at 24 h and 36 h postirradiation in both cell lines. This coincided with accumulation of the G2-M fraction revealed by flow cytometry (Table 1). However, there was no difference in cyclin B1 expression between TE8-CDC25B and the vector control. Tyrosine-phosphorylated CDC2, which was detected by antiphosphotyro-rosine antibody in CDC2 immunoprecipitates, gradually increased after radiation in both cell lines, whereas the amount was higher in TE8-neo than in TE8-CDC25B throughout the period (Fig. 4b). The protein amount of CDC25B immediately and remarkably decreased after radiation, as more than 80% of the CDC25B protein disappeared in both cell lines at 12 h postradiation and, thereafter, recovered gradually until 48 h. In addition, TE8-CDC25B retained higher CDC25B expression after irradiation than did the vector control, especially twice as much at 12 h. Both cell lines displayed similar amounts of CDC25C protein, which slightly decreased at 12 h postradiation and soon recovered at 24 h postradiation. Thus, after radiation, there was no difference in the expression of cell cycle regulatory proteins between TE8-CDC25B and TE8-neo except for CDC25B and tyrosine-phosphorylated CDC2.

Effect of CDC25B Overexpression on CDC2 Kinase Activity after Radiation. More than 60% of the cells were accumulated in the S phase by the double thymidine block method, then were released and entered the G2 phase after 8–12 h. During this period, there was no significant difference among cell lines for the amount of synchronized cells and cell cycle progression. Next, TE8-CDC25B and TE8-neo were subjected to 10-Gy radiation 4 h after release from the double thymidine block and just before entering the G2 phase. In both cell lines, the CDC2 kinase activity was immediately reduced after radiation (observed at 0.5, 1, and 3 h) and recovered after about 6–10 h postradiation (Fig. 5a). During this period, TE8-CDC25B always showed higher expression of CDC25B than TE8-neo, and the amount of CDC25B protein decreased at 6 h postradiation in both cell lines (Fig. 5b). It was notable that the CDC2 kinase activity was sustained more in TE8-CDC25B than in TE8-neo, especially in the early-phase postradiation. For example, the CDC2 kinase activity was twice as much after 0.5 h in TE8-CDC25B. In contrast, the CDC2 kinase activity was weak in asynchronous cells, and, therefore, its inhibition after radiation and the difference between TE8-CDC25B and TE8-neo were difficult to evaluate. Interestingly, in asynchronous cells, the CDC2 kinase activity increased in the late phase after radiation (at 12–36 h postradiation) along with G2-M accumulation, although no significant difference was observed among cell lines.

DISCUSSION

In this study, we have shown that CDC25B overexpression results in suppression of G2-M arrest, induction of apoptosis and consequently high sensitivity for radiation therapy. This is the first report to experimentally reveal the implication of CDC25B in radiation sensitivity, which should be very useful information for clinical cancer treatment by radiation therapy.

We used CDC25B2 cDNA for this study, because it was expected to offer the highest phosphatase activity. CDC25B has three splicing variants: CDC25B1, CDC25B2, and CDC25B3 (34, 35). Structurally, CDC25B3 is a full-length 580-residue form, whereas CDC25B1 lacks 14 residues and CDC25B2 lacks 42 residues. CDC25B2 can activate CDC2/cyclin B1 and induce mitosis more efficiently than the others (34). CDC25C is also involved in the regulation of the G2-M checkpoint. Overexpression of CDC25C together with cyclin B1 shortens the G2 phase and induces premature mitosis; however, it does so less efficiently than does the overexpression of CDC25B (36). Moreover, it is discouraging that CDC25C is not overexpressed in human cancers. Our previous study using human esophageal cancer samples has shown that among CDC25A, CDC25B, and CDC25C, only CDC25B expression has a close relation to radiation sensitivity (15). Therefore, we used, not CDC25C, but CDC25B in this study. Consistently, there was no difference in CDC25C expression between TE8-CDC25B and TE8-neo clones in this study.

In our system, overexpression of CDC25B was sufficient to sustain the CDC2 kinase activity and override G2-M arrest after DNA damage. As shown in Fig. 4, ~80% of CDC25B proteins disappeared at 12 h after radiation. This is probably because Chk1-phosphorylated CDC25B is rapidly sequestered by 14-3-3 protein. Although a decrease in CDC25B mRNA is also reported after radiation (37), this is not likely to happen in our experiment because exogenous CDC25B mRNA did not change after radiation (data not shown). In TE8-CDC25B, a small amount of CDC25B that escaped from the Chk1/
14-3-3 protein system and was still more than the amount in the vector control, could lead to progression of the G2-M phase. On the other hand, overexpression of CDC25B had little effect on G2-M progression in the absence of radiation. In the TE8 esophageal cancer cell line, the amount of CDC25B was already enough to oscillate G2-M phase, and no further effect was obtained by its overexpression. Thus, the protein amount of CDC25B is strictly regulated, and CDC25B would be one of the most important target molecules for radiation-induced G2-M arrest.

There was a discrepancy in the time course between suppression of CDC2 kinase activity (until 6 h) and G2-M arrest (until 36 h). Moreover, the CDC2 kinase activity in asynchronized cells, although much weaker than in synchronized cells in the late G2 phase, was slightly increased accompanying G2-M arrest. These observations suggest the existence of another mechanism for G2-M arrest other than the reduction of CDC2 kinase activity or CDC25B protein. HeLa cells treated with etoposide and caffeine are unable to enter mitosis despite high CDC2/cyclin B1 activity (38), because of interference of nuclear translocation of CDC2/cyclin B1 complex. Cyclin B1 has a nuclear export signal that is regulated by CRM1 (39, 40). In addition, nuclear translocation of CDC25B is required for G2-M progression, nuclear export signal that is regulated by CRM1 (39, 40). In addition, nuclear translocation of CDC2/cyclin B1 complex. Cyclin B1 has a nuclear export signal (41, 42). Taken together, the nuclear transporting system might be involved in radiation-induced G2-M arrest and, therefore, should be investigated in the future.

The mechanism of the cell with DNA damage to undergo apoptosis at the G2-M checkpoint has not been elucidated. Disorders of mismatch repair gene are known to permit DNA-damaged cells to escape apoptosis (43, 44). Thus, the DNA repair system might recognize DNA damage that cannot be restored and trigger the apoptosis switch. In this experiment, apoptosis emerged just after release from G2-M arrest, and a lower dose of irradiation induced G2-M arrest but not apoptosis. These findings suggest that the balance between the extent of DNA damage and the duration of G2-M arrest may decide the fate of irradiated cells: survival or apoptosis.

Many experiments have revealed another pathway through p53 downstream of ATM, which directly stimulates the apoptotic signal, such as bax (45). After radiation, we found that both p53 and CDC25B were independently associated with radiation sensitivity in human esophageal cancers (15). p53 also regulates the cell cycle; however, it is more strongly associated with G1 arrest than with G2-M arrest. In the present study, we used TE8 esophageal cancer cells with mutant p53 and obtained similar results by CDC25B transfection using other cell lines with wild (TE3) and mutated (TT) p53 (data not shown). Thus, the G2-M checkpoint seems to be preserved regardless of the p53 status. This is very important in clinical cancer treatment because p53 is frequently mutated in human cancers. For example, p53 mutation was observed in more than 50% of advanced esophageal cancers (46, 47).

According to the results of this study, radiation therapy should be an effective strategy against cancer cells with CDC25B overexpression. Furthermore, it is encouraging that some compounds that abrogate G2-M arrest and stimulate apoptosis after DNA damaging treatments are clinically available. For example, hydroxyxstatasporine (UCN-01) inhibits Chk1 followed by inactivation of weel and activation of CDC25 (48, 49). Caffeine has been reported to increase the radiosensitivity of p53-deficient cancer cells by CDC2 activation (50). Recently, short peptides that inhibit both Chk1 and Chk2 activates have been reported to specifically abrogate DNA-damage-induced G2-M checkpoint and to sensitize cancer cells to DNA-damaging agents (51). These compounds might override radiation resistances in the cancers without CDC25B overexpression. Thus, study of the G2-M checkpoint and CDC25B overexpression should yield useful findings for clinical applications.

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