Sensitization of Cancer Cells to DNA Damage-induced Cell Death by Specific Cell Cycle G₂ Checkpoint Abrogation¹

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Abstract

We devised two short peptides corresponding to amino acids 211–221 of human Cdc25C fused with a part of HIV-1-TAT. These peptides inhibited hChk1 and Chk2/HuCds1 kinase activity in vitro and specifically abrogated the G₂ checkpoint in vivo. These peptides sensitized p53-defective cancer cell lines to DNA-damaging agents to death without obvious cytotoxic effect on normal cells. Our results clearly indicate that the specific abrogation of the cell cycle G₂ checkpoint is a feasible strategy for cancer therapy, and hChk1 and Chk2/HuCds1 are proper targets for that purpose.

Introduction

Most of the cancer cells have mutations in genes involved in the G₁ checkpoint such as impaired tumor suppressor genes including p53, Rb, p16INK4a, and p19ARF or overexpression of relevant oncogenes including MDM-2 and cyclin D (1). In addition to these, excessive growth factor signaling caused by the overexpression of growth factors, together with the downstream signal-transducing molecules would give rise to cell transformation by overriding the G₁ checkpoint. Only exceptionally, some cancer cells possess the disrupted G₂ checkpoint instead of G₁ checkpoint (2). This indicates the relative importance of G₁ checkpoint compared with the G₂ checkpoint for the normal human cell cycle. These checkpoint dysfunctions confer cells with the ability to accumulate mutations that eventually lead to carcinogenesis (3), although excessive accumulation of mutations would be lethal to the cells. Interestingly, the G₂ checkpoint is usually retained in the cancer cells with impaired G₁ checkpoint. If the G₂ checkpoint was selectively disrupted by the treatment, the cancer cells with the impaired G₁ checkpoint would become more sensitive to the DNA-damaging treatment compared with normal cells because normal cells still retain intact G₁ checkpoint. For example, relatively nonspecific G₂ checkpoint dysfunctions by caffeine or UCN-01⁴ were reported to be effective in sensitizing the p53-defective cancer cells to DNA damage (4, 5). However, the effects of sensitizing cancer cells by these compounds were subtle, and in some reports, caffeine made cells even more resistant to genotoxic treatments (6), probably because of other irrelevant effects of these compounds to the cells (7).

The mechanism that promotes the cell cycle G₂ arrest after DNA damage is conserved among species from yeast to human. In the presence of damaged DNA, Cdc2/Cyclin B is kept inactive because of inhibitory phosphorylation of threonine-14 and tyrosine-15 residues on Cdc2 (8). At the onset of mitosis, the dual phosphatase Cdc25 removes these inhibitory phosphates and thereby activates Cdc2/Cyclin B (9).

In fission yeast, the protein kinase Chk1 is required for the cell cycle arrest in response to damaged DNA (10). Chk1 acts downstream of several rad gene products and is modified by the phosphorylation upon DNA damage (11). The kinases Rad53 of budding yeast and Cds1 of fission yeast are known to conduct signals from unreplicated DNA (12). It appears that there is some redundancy between Chk1 and Cds1 because elimination of both Chk1 and Cds1 was culminated in disruption of G₂ arrest induced by unreplicated DNA (13). Interestingly, both Chk1 and Cds1 phosphorylate Cdc25 and promote Rad24 binding to Cdc25 (14, 15), which sequesters Cdc25 to cytosol and prevents Cdc2/Cyclin B activation (16). Therefore, Cdc25 appears to be a common target of these kinases and presumably an indispensable factor in G₂ checkpoint.

In human, both hChk1 (17, 18), a human homologue of fission yeast Chk1, and Chk2/HuCds1 (19), a human homologue of the budding yeast Rad53 and fission yeast Cds1 at serine-216, a critical regulatory site, in response to DNA damage (17–20). This phosphorylation creates a binding site for small acidic proteins 14-3-3, human homologues of Rad24 and Rad52 of fission yeast (20). Although serine-216 of Cdc25C can be constitutively phosphorylated by kinase(s) such as C-TAK1/Kp78/MARK3 (21–23), the regulatory role of this phosphorylation was clearly indicated by the fact that substitution of serine-216 on Cdc25C to alanine disrupted cell cycle G₂ arrest in human cells (20).

Here we demonstrate a novel finding that inactivation of hChk1 and Chk2/HuCds1 kinase activities can be achieved by small peptides corresponding to amino acids 211–221 of human Cdc25C. We also show that these peptides can efficiently disrupt the cell cycle G₂ checkpoint that is activated by DNA damage and thus sensitize cancer cells but not normal cells to anticancer reagents.

Materials and Methods

Chemicals and Reagents. Bleomycin and colchicine were purchased from Wako Pure Chemical Co. (Osaka, Japan). Hydroxyurea was purchased from Sigma Chemical Co. (St. Louis, MO). These chemicals were dissolved in distilled H₂O to 10, 5, and 50 mg/ml, respectively, and stored at 4°C. Antibodies against 14-3-3-B were purchased from Santa Cruz Biotechnology (Santa Cruz, CA), and antirabbit IgG horseradish peroxidase-conjugated secondary antibodies were purchased from Amersham Life Sciences (Arlington Heights, IL). Antibodies against HA and c-myc and protein G-Sepharose were purchased from Santa Cruz Biotechnology and Amersham Pharmacia Biotech (Uppsala, Sweden), respectively.

Cell Culture and Plasmid. A human T-cell leukemia-derived cell line, Jurkat, was cultured in RPMI 1640 (Sigma) supplemented with 10% FCS (IBL, Gunma, Japan) at 37°C/5% CO₂. Human pancreatic epitheloid carcinoma-derived cell lines, MiaPaCa2 and Panc1, were cultured in Eagle’s MEM (Iwaki, Chiba, Japan) and DMEM with 4 mM l-glucose (Sigma) and 1.0

¹ The abbreviations used are: UCN-01, 7-hydroxystaurosporine; PHA, phytohemagglutinin; HA, hemagglutinin; GST, glutathione S-transferase; FACS, fluorescence-activated cell sorter.

Received 8/11/99; accepted 10/18/99.

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TAT-S216 peptide was synthesized so that it contained an NH₂-terminal 11 amino acid TAT protein transduction domain (YGRKKRRQRRR; Ref. 24), followed by corresponding amino acids 211–221 derived from the Cdc25C amino acid sequence (S216; LYRSPSM PNEL). Serine-216 residue was changed to alanine in TAT-S216A (S216A; LYRSPSM PNEL). The Cdc25C portion was partially deleted and substituted with glycine in TAT-Control (GGRSPAMEP). All peptides were synthesized by Sawady Technology Co. (Tokyo, Japan).

### Purification of Recombinant GST-Cdc25C Proteins.

Escherichia coli DH5α cells were transformed by GST-Cdc25C (200–256) plasmid. The cells were incubated with 0.1 mM isopropyl β-d-thiogalactoside for 2 h, harvested, and lysed with a buffer containing 50 mM Tris-HCl (pH 8.0), 100 mM NaCl, 0.5% NP40, 5 μg/ml aprotinin, 5 μg/ml pepstatin A, and 5 μg/ml leupeptin. The lysate was sonicated, centrifuged for clarification, and incubated with glutathione-Sepharose 4B beads for 1 h at 4°C and washed five times.

### Kinase Assay.

HA-tagged hChk1 and c-myc-tagged Chk2/HuCds1 expressed in insect cells using recombinant baculovirus (Ref. 18) were purified by immunoprecipitation using anti-HA or anti-c-myc antibodies and protein G-Sepharose. The lysate was sonicated, centrifuged for clarification, and incubated with 0.1 mM isopropyl β-d-thiogalactoside for 2 h, harvested, and lysed with a buffer containing 50 mM Tris-HCl (pH 8.0), 100 mM NaCl, 0.5% NP40, 5 μg/ml aprotinin, 5 μg/ml pepstatin A, and 5 μg/ml leupeptin. The lysate was sonicated, centrifuged for clarification, and incubated with glutathione-Sepharose 4B beads for 1 h at 4°C and washed five times.

### Cell Cycle Analysis.

The cell cycle status of the cells treated with peptides and/or bleomycin or colchicine was analyzed by FACS (2). Two million Jurkat cells were resuspended and incubated in 300 μl of Krshan’s solution (0.1% sodium citrate, 50 μg/ml propidium iodide, 20 μg/ml RNase A, and 0.5% NP40; Ref. 2) for 1 h at 4°C and analyzed by FACSscan (Becton Dickinson, Mountain View, CA) with the program CELLQuest (Becton Dickinson).

### Histone H1 Kinase Assay.

Ten million Jurkat cells were treated with hydroxyurea (100 μg/ml), bleomycin (10 μg/ml), or colchicine (5 μg/ml) with or without addition of TAT-S216A, TAT-S216, or TAT-Control (10 μM) for 6 h. The cells were washed in cold PBS and lysed at 4°C in 1 ml of buffer A (50 mM Tris pH8, 2 mM DTT, 1 mM MgCl₂, and 100 μM of [γ-32P]ATP (Amersham; 6000 Ci/mmol) plus purified 1 μM GST-Cdc25C or 10 μM Cdc25c peptide (amino acids 211–221 of Cdc25C; LYRSPSM PNEL; Sawady Technology Co.) substrates at 30°C for 15 min in the presence of 10 μM TAT-S216, TAT-S216A, or TAT-Control. After the reaction, samples were separated in 12 or 15% SDS-PAGE and autoradiographed to detect GST-Cdc25C or peptide phosphorylation.

### Cell Cytotoxicity Assay.

MIAPaCa2 and PANC1 cells (3 x 10⁵/well) were plated in 96-well microtiter plates. After an overnight adherence, cells were treated with bleomycin (10 μg/ml) with or without the indicated TAT-peptides at various time points up to 96 h. Cytotoxicity and cell survival were determined by the Trypan Blue exclusion assay (Cell Proliferation Kit II; Boehringer Mannheim, Mannheim, Germany), which was done according to the company’s protocol (25).

### Results

**TAT-S216 and TAT-S216A Peptides Inhibit hChk1 and Chk2/HuCds1 Kinase Activities.** In our initial attempts to inhibit hChk1 and Chk2/HuCds1 kinase activities and to abrogate DNA damage-induced G₂ arrest, we generated synthetic peptide consisted with amino acids 211–221 of Cdc25C (TAT-S216). TAT-S216A peptide in which serine-216 was substituted by alanine to stabilize the transient status of its interaction with hChk1 and Chk2/HuCds1 (Fig. 1A). To efficiently transduce these peptides into cells, a part of the HIV-1 TAT peptide sequence was included (Ref. 24; Fig. 1A). This sequence is known to facilitate the uptake of heterologous proteins across the cell membrane. As a control peptide, part of the Cdc25C portion of this peptide was deleted (TAT-Control).

As shown in Fig. 1B, hChk1 was capable of phosphorylating a Cdc25C protein (residues 200–256) fused to GST. Serine-216 on Cdc25C is the major phosphorylation site of this fusion protein in vivo (14, 17, 20). In Fig. 1B, both TAT-S216 and TAT-S216A inhibited the phosphorylation of Cdc25C by baculovirus produced hChk1. TAT-S216 but not TAT-S216A was efficiently phosphorylated by hChk1, suggesting that serine-216 on TAT-S216 was phosphorylated by hChk1, and TAT-S216 would competitively inhibit substrate phosphorylation at excess molar ratio if present in great enough quantity. TAT-Control peptide did not inhibit hChk1 kinase activity (data not shown). As shown in Fig. 1C, TAT-S216A significantly inhibited...
phosphorylation of Cdc25C peptide mediated by hChk1 and Chk2/HuCds1, even at a low stoichiometry (at four times more molar excess of TAT-S216A peptide against substrate Cdc25C peptide).

Abrogation of DNA Damage-induced G2 Checkpoint by TAT-S216 and TAT-S216A Peptides. We then analyzed the cell cycle status of the cells treated with TAT-S216A or TAT-S216 upon the DNA damage-induced G2 arrest by FACS analysis. Histone H1 kinase activities of these cells were simultaneously monitored. Jurkat cells arrested exclusively at G2 by bleomycin (10 μg/ml) treatment, because it does not have functional p53. As shown in Fig. 2A, G2 arrest was completely abrogated by the addition of TAT-S216A or TAT-S216 in response to bleomycin. G2 arrest was abrogated at any time point between 12 and 48 h by the treatment with TAT-S216A or TAT-S216 (data not shown). Jurkat cells treated with bleomycin, together with TAT-Control, arrested at G2 similarly to the cells treated with bleomycin alone. We also observed that either TAT-S216A or TAT-S216 also abrogated G2 arrest induced by γ-irradiation and cisplatin (γ-irradiation, 5 Gy; cisplatin, 1 μg/ml; for 1 h treatment; data not shown). To further analyze the effect of these peptides on G2-M transition, histone H1 kinase activity was monitored. Consistently with the above findings, although histone H1 kinase activity was decreased by the treatment with bleomycin or hydroxyurea, it was unchanged or rather increased by the treatment with bleomycin in the presence of TAT-S216A or TAT-S216 (Fig. 2B). In the presence of TAT-Control peptide, the bleomycin treatment did not affect with H1 kinase activity (data not shown). As shown in Fig. 2C, the M-phase...
arrest of Jurkat cells induced by colchicine was not affected by the addition of TAT-S216 or TAT-S216A. These results strongly suggested that TAT-S216A and TAT-S216 specifically abrogated the DNA damage-activated cell cycle G2 checkpoint by inhibiting hChk1 and/or Chk2/HuCds1 kinase activities.

Sensitization of Jurkat Cells to Bleomycin-induced Cell Death by TAT-S216A and TAT-S216 Peptides. We then examined the effect of TAT-S216A and TAT-S216 on the cell death induced by bleomycin. As shown in Fig. 3A, the addition of TAT-S216A and TAT-S216 efficiently sensitized Jurkat cells to the bleomycin-induced cell death. Whereas bleomycin treatment at 5 or 10 μg/ml killed Jurkat cells by only 27–30%, the addition of 10 μM TAT-216A or TAT-S216 killed Jurkat cells by nearly 80%. In contrast, these peptides by themselves did not show any significant cytotoxicity. In addition, a control peptide TAT-Control did not affect the viability of bleomycin-treated Jurkat cells (data not shown). Moreover, as expected from the result in Fig. 2C, either TAT-S216A or TAT-S216 did not affect the cytotoxicity by colchicine (Fig. 3B). This observation indicates that the cell death induced by these peptides in the presence of bleomycin was not attributable to the nonspecific cytotoxic effect.

TAT-S216 and TAT-S216A Peptides Did Not Affect the Viability of Normal Cells. To confirm the specificity of the effect of these peptides on cancer cells in which the G2 checkpoint is abrogated, we investigated the effect of these peptides on normal human cells. We prepared mitogen-activated normal human T lymphocytes (PHA blasts) by stimulating peripheral blood mononuclear cells obtained by themselves did not show any significant cytotoxicity. In addition, a control peptide TAT-Control did not affect the viability of bleomycin-treated Jurkat cells (data not shown). Moreover, as expected from the result in Fig. 2C, either TAT-S216A or TAT-S216 did not affect the cytotoxicity by colchicine (Fig. 3B). This observation indicates that the cell death induced by these peptides in the presence of bleomycin was not attributable to the nonspecific cytotoxic effect.

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from a healthy donor with PHA for 1 week. These cells were treated with bleomycin (5 and 10 μg/ml) in the presence or absence of either TAT-S216A or TAT-S216. As shown in Fig. 3C, these peptides did not augment the cytotoxic effect of bleomycin, although these cells replicated as fast as Jurkat cells. As shown in Fig. 3D, PHA blasts treated with bleomycin (5 μg/ml) arrested at G1 and S phase but not G2, presumably because of the activity of wild-type p53. When these cells were treated with TAT-S216 or TAT-S216A in addition to bleomycin, no further alteration of cell cycle pattern was observed.

Sensitization of Pancreatic Cancer Cells to the Bleomycin-Induced Cell Death by TAT-S216A and TAT-S216 Peptides. We then examined the effect of these peptides on two other p53-defective cancer cell lines, MiaPaCa2 and Panc1 cells. Although these pancreatic cancer cells are known to be resistant to various anticancer reagents, these cells could also be sensitized to the bleomycin-induced cell death by TAT-S216A and TAT-S216 (Fig. 4). Similarly, these peptides could sensitize these cells to the cell death induced by other DNA-damaging agents including cisplatin and γ-irradiation (data not shown).

Discussion

The effects of abrogating cell cycle G2 checkpoint on the sensitization of the G2-abrogated cancer cells to cell death induced by DNA-damaging agents have long been investigated (4–7). For example, effects of caffeine have been studied extensively (4, 6). However, these results were considered to be controversial because of its various pharmacological actions other than its potential effects on cell cycle checkpoint disruption (7). Similarly, the use of UCN-01, initially developed as a protein kinase C inhibitor, has been claimed to work by disrupting the G2 checkpoint (5). However, UCN-01 also shows a variety of activity other than the G2 checkpoint abrogation. Thus, it is still controversial whether the abrogation of the G2 checkpoint is effective to sensitize cancer cells to the DNA damage-induced cell death.

In this report, we have demonstrated for the first time that short peptides that inhibit both hChk1 and Chk2/HuCdS1 kinase activities specifically abrogated DNA damage-induced G2 checkpoint. We also demonstrated that the specific abrogation of the G2 checkpoint sensitized cancer cells to bleomycin, a DNA-damaging agent, without sensitizing normal cells (PHA blasts) to the DNA damage-induced cell death. Because the cell cycle pattern and the viability of normal cells (PHA blasts) were not affected by the peptides containing this structural moiety, application of these inhibitory peptide would not have any effect in killing normal cells, unlike other anticancer drugs. Whereas these peptides by themselves are candidates for efficient anticancer agents, when used with known DNA-damaging agents, smaller molecular compounds that can inhibit both hChk1 and Chk2/HuCdS1 kinase activities are needed for more cost-effective and practical candidates for anticancer medicine. We believe that these findings should facilitate the development of a novel therapy against intractable cancers that are resistant to conventional anticancer therapies.

Acknowledgments

We thank Dr. M. Nakanishi for the kind gift of HA-hChk1, c-myc-Chk2/ 
HuCdS1, and GST-Chd25C plasmid. We also thank K. Aoyama, H. Sato, and M. Kimura for assistance.

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