Inhibition of Cell Death and Induction of G2 Arrest Accumulation in Human Ovarian Clear Cells by HNF-1β Transcription Factor

Chemosensitivity Is Regulated by Checkpoint Kinase CHK1

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Objective: Appropriate cell cycle checkpoints are essential for the maintenance of normal cells and chemosensitivity of cancer cells. Clear cell adenocarcinoma (CCA) of the ovary is highly resistant to chemotherapy. Hepatocyte nuclear factor-1β (HNF-1β) is known to be overexpressed in CCA, but its role and clinical significance is unclear. We investigated the role of HNF-1β in regulation of the cell cycle in CCA.

Methods: To clarify the effects of HNF-1β on cell cycle checkpoints, we compared the cell cycle distribution and the expression of key proteins involved in CCA cells in which HNF-1β had been stably knocked down and in vector-control cell lines after treatment with bleomycin. HNF-1β (+) cells were arrested in G2 phase because of DNA damage.

Results: HNF-1β (−) cells died because of a checkpoint mechanism. G2 arrest of HNF-1β (+) cells resulted from sustained CHK1 activation, a protein that plays a major role in the checkpoint mechanism. HNF-1β (+) cells were treated with a CHK1 inhibitor after bleomycin treatment. Flow cytometric analysis of the cell cycle demonstrated that DNA damage-induced G2-arrested cells were released from the checkpoint and killed by a CHK1 inhibitor.

Conclusions: The chemoresistance of CCA may be due to aberrant retention of the G2 checkpoint through overexpression of HNF-1β. This is the first study demonstrating cell cycle regulation and chemosensitization by a CHK1 inhibitor in CCA.

Key Words: Transcription factors, DNA damage response, Cell cycle, Checkpoint control, Chemoresistance

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Endometriosis.3 Endometriosis is a common gynecological condition and affects an estimated 10% of women of reproductive age.4 The frequency of CCA is thought to be 5% to 10% of all epithelial ovarian cancer in Western countries, and it is higher (~20%) in Japan.5-10 It is readily treatable during the early stages of development, but prognosis is grave once the disease metastasizes because paclitaxel and carboplatin combination therapy is not efficacious for CCA.5 Currently available therapies are not effective in preventing or curing metastatic spread and morbidity in patients with this cancer.

Free iron in ovarian endometriotic cysts has been shown to induce oxidative stress, inflammatory responses, cellular toxicity and tissue injuries, genetic changes, and epigenetic alterations in target cells and tissues.11 Among ovarian cancers, CCA has been recognized as a distinct clinicopathological entity because of its frequent concurrence with endometriotic lesions and its high chemoresistance, resulting in poor prognosis of late stage tumors.6 However, the molecular events involved in this transformation have not been clarified. Recent biochemical studies based on genome-wide expression analysis technology have noted specific expression of a transcription factor, HNF-1β. Tsuchiya et al12 reported that treatment of CCA cells with siRNA against HNF-1β increased apoptosis. This suggested that HNF-1β is involved in promotion and progression of CCA.

Twenty-two (40.7%) of 54 genes predominantly identified in CCA are downstream targets of HNF-1β.13-15 The HNF-1β-dependent pathway may provide new insights into the regulation of resistance to anticancer agents. Appropriate cell cycle checkpoints are essential for the maintenance of normal cells and chemosensitivity of cancer cells.16 In the present study, these observations were extended, and the mechanism and functional significance of HNF-1β-induced cell cycle progression was determined.

MATERIALS AND METHODS

Cell Lines

Clear cell adenocarcinoma cell lines, TU-OC-1, KOC7c, RMG-1 and RMG-2 were kindly provided by Dr H. Itamochi (Tottori University, Tottori, Japan). TOV-21G was purchased from American Type Culture Collection (ATCC, Manassas, Va). MCAS, RMUG-L, and RMUG-S were purchased from the Japan Health Sciences Foundation. These cells were maintained in DMEM/F12 (Invitrogen, Carlsbad, Calif), supplemented with 10% fetal bovine serum (FBS) and 100 U/mL penicillin/streptomycin (Invitrogen). Clear cell adenocarcinoma cell line ES2 was purchased from ATCC, and maintained in McCoy 5A medium containing 10% FBS and 100 U/mL penicillin/streptomycin. Cervical cancer cell line HeLa, and ovarian adenocarcinoma cell line SKOV-3 were purchased from ATCC and cultured in DMEM/F12 containing 10% FBS and 100 U/mL penicillin/streptomycin.

Protein Preparation

Cells were washed after treatment with ice-cold PBS followed by addition of 10% TCA, and kept on ice for 15 minutes. Samples were freed from the substrate using a cell scraper, collected into ice-cooled microfuge tubes, and centrifuged for 10 minutes at 10,000g. The protein pellet was then washed with ice-cold PBS and centrifuged twice for 10 minutes at 10,000g. The final protein pellet was dissolved in SDS-PAGE loading buffer (50 mM Tris-HCl (pH 6.8), 2% SDS, 10% glycerol, 0.1% BPB, and 0.1 M DTT) at 100°C for 5 minutes.

Western Blot Analysis

Protein samples were separated on SDS-polyacrylamide gels and then transferred to PVDF membranes. Blots were blocked with Blocking One-P (Nacalai Tesque Inc, Kyoto, Japan) at room temperature for 20 minutes and were then probed with primary antibodies against HNF-1β (1:1000; BD Biosciences, San Diego, Calif), phospho-CHK1 (1:1000; Cell Signaling Technology, Inc, Danvers, Mass), CHK1 (1:1000; Cell Signaling Technology, Inc), and β-actin (1:500; Santa Cruz Biootechnology, Santa Cruz, Calif) at 4°C overnight. Horseradish peroxidase-conjugated secondary antibody was used at a dilution of 1:5000. Proteins were visualized using enhanced chemiluminescence and normalized to β-actin.

Stable Knockdown of HNF-1β

An RNA interference vector for the HNF-1β gene (HuSh 29mer shRNA Constructs against TCF2; OriGene Technologies, Rockville, Md) was transfected into TU-OC-1 cells using Lipofectamine LTX (Invitrogen) according to the manufacturer’s protocol. After transfection, a selective culture using 0.25 μg/mL puromycin (Invitrogen) was performed to establish a clone with stable knockdown of HNF-1β (TU-OC-1-shHNF-1β).

siRNA and Transfection

siRNA (Qiagen, Valencia, Calif) against HNF-1β (TCF2), Hs_TCF2_7, was used. AllStars Negative Control siRNA (Qiagen) was used as a control siRNA. Cells were seeded in 6-well plates at a density of 2 × 10⁵ cells/well and cultured for 24 hours. Cells were washed with PBS and transfected with 30 nM siRNA using HiPerFect Transfection Reagent (Qiagen) in accordance with the manufacturer’s instructions.

Cell Cycle Analysis Using Propidium Iodide

Intracellular DNA content was analyzed by fixing cells in 70% ethanol at −20°C for several hours. Cells were resuspended in PBS containing RNase at 0.1 mg/mL. Samples were incubated at 37°C for 15 minutes, and propidium iodide was added to a final concentration of 25 μg/mL. Samples were processed using a Cytomics FC500 (Beckman Coulter, Brea, Calif).

Analysis of DNA Damage Checkpoint

To clarify the effects of HNF-1β on cell cycle checkpoints, cell cycle distribution and the expression of key proteins were examined in TU-OC-1-shHNF-1β and vector-control cell lines treated with 5 μM cisplatin, 0.5 μg/mL nocodazole, or 42 μM bleomycin to induce DNA damage.

Reagents

Cisplatin and bleomycin were purchased from Nippon Kayaku Co, Ltd (Tokyo, Japan). Nocodazole was purchased from Sigma Chemical (St Louis, Mo). AZD7762, CHK1 inhibitor (Selleck Chemicals, Houston, Tex), was dissolved in DMSO. Cells were treated with 50 nM AZD7762 after bleomycin treatment.
RESULTS

Our previous immunohistochemistry experiments showed that HNF-1β was significantly up-regulated in 90% of CCA samples from 29 patients.

To clarify the expression of HNF-1β in CCA cell lines, a panel of 10 cell lines that included human CCA cells was analyzed for HNF-1β protein alterations. As shown in Figure 1, variable levels of HNF-1β protein were expressed in 6 cell lines. HNF-1β protein was completely absent in ES2 cells, and RMG-1 and RMG-2 expressed very high HNF-1β levels. TU-OC-1, TOV21G, and KOC-7d expressed moderate levels of HNF-1β protein. Western blot analysis showed 80% of the CCA cell lines expressed high levels of HNF-1β, whereas no expression was detected in the other cell lines.

HNF-1β was also overexpressed in CCA cell lines, in accordance with previous immunological and microarray studies in CCA.

Effects of HNF-1β on Cell Cycle Checkpoints

Clear cell adenocarcinoma has been recognized to show resistance to anticancer agents due to abnormal cell cycle regulation.17 To clarify the effects of HNF-1β on the cell cycle checkpoints, asynchronous TU-OC-1-shHNF-1β and vector-control cell lines were treated with various anticancer drugs.

First, we investigated the effect of HNF-1β on cisplatin-induced DNA damage checkpoints. After addition of cisplatin, both HNF-1β (+) and (-) cells were mainly arrested at S-phase, indicating no difference in response to cisplatin-induced checkpoint (Fig. 2A).

Next, mitotic checkpoints were examined using nocodazole, which inhibits spindle-kinetochores interaction by microtubule disruption. Addition of nocodazole resulted in the arrest of both HNF-1β (+) and (-) cells in mitosis, indicating no difference in response to the mitotic checkpoint (Fig. 2B).

Finally, to investigate the response to the G2 checkpoint, HNF-1β (+) and (-) cells were treated with bleomycin, which is known to arrest the cell cycle at G2. Up to 12 hours, both HNF-1β (+) and (-) cells accumulated in G2 at similar levels. Although HNF-1β (-) cells exited from G2 arrest with subsequent increase in cell death, HNF-1β (+) cells remained arrested in G2, indicating that HNF-1β induced aberrant retention of the G2 checkpoint to result in resistance to bleomycin (Fig. 2C). These data indicated that the anticancer drug resistance of CCA may be caused by aberrant G2 arrest due to HNF-1β overexpression.

Cell Cycle Analysis After the Addition of Bleomycin in HNF-1β siRNA-Transfected Cells

To demonstrate that the G2 arrest sustained by HNF-1β was not restricted to the TU-OC-1 line, siRNA was introduced into TOV-21G, a CCA line, to transiently knock down HNF-1β in the same experimental system. As observed with the TU-OC-1 line, bleomycin sustained G2 arrest and increased cell death in the HNF-1β (-) cells (Fig. 2D,E).

An Abnormal G2 Checkpoint System in HNF-1β-Overexpressing Cells

CHK1 kinase acts downstream of ATM/ATR kinase and plays an important role in the G2 checkpoint. Therefore, to clarify the effect of HNF-1β on the function of CHK1, we investigated the phosphorylation status of CHK1 at serine296 (active form) in HNF-1β (+) and (-) cells after bleomycin treatment. The basal level of CHK1 protein was expressed at a comparable level in HNF-1β (+) and (-) cells (Fig. 3A). Although phosphorylated CHK1 was down-regulated in HNF-1β (-) cells after 24 hours, CHK1 was increasingly up-regulated and maintained at high levels in HNF-1β (+) (Fig. 3B).

These data indicated that the G2 arrest of HNF-1β (+) cells resulted from sustained CHK1 activation.

Anticancer Agents Combined With CHK1 Inhibitors May Sensitize CCA

Zhang et al18 recently reported that alterations in CHK1 expression regulated by ubiquitination and degradation represent a common mechanism for anticancer therapy resistance.
Currently, the effects of CHK1 inhibitors on various cancers are being investigated in clinical trials. Cells lacking intact G1 checkpoints through inactivation of p53 are particularly dependent on S and G2/M checkpoints and are therefore expected to be more sensitive to chemotherapeutic treatment in the presence of a CHK1 inhibitor, whereas normal cells with functional G1 checkpoints are predicted to undergo less cell death.19

Our study suggested that anticancer drug resistance of CCA lines may be caused by sustained activation of the G2 checkpoint by HNF-1A. Thus, we examined whether bleomycin combined with a CHK1 inhibitor increased anticancer drug resistance. A CHK1 inhibitor added 24 hours after the addition of bleomycin abrogated phospho-CHK1 (Fig. 4A). Flow cytometric analysis of the cell cycle demonstrated that G2-arrested cells due to DNA damage were released from the checkpoint and killed by a CHK1 inhibitor (Fig. 4B). Anticancer drugs combined with CHK1 inhibitors may improve the chemosensitivity of CCA.

**DISCUSSION**

Several sporadic cancers, including hepatocellular, renal, and cervical cancer, experience a type of oxidative stress-induced genetic instability. Clear cell adenocarcinoma is a model of persistent oxidative stress that damages nucleotides. It has been reported previously that the HNF-1β-dependent pathway may provide new insights into the regulation of detoxification and resistance to anticancer agents.12 A redox-sensitive subset of CCA genes linked to oxidative and detoxification pathways was identified and associated with HNF-1β-specific downstream targets.13-15 Furthermore, Itamochi et al17 previously found an association between reduced proliferation of CCA cells and chemoresistance. These data allow us to speculate that HNF-1A plays an important role in regulating inappropriate cell cycle progression in CCA cells characterized by damaged DNA.

Cell cycle checkpoints monitor normal cell cycle progression and discontinue cell cycle progression when any abnormality or defect occurs. The G1/S, G2/M, and spindle formation checkpoints have been analyzed in detail. These checkpoints play fundamental roles by transmitting genetic information to daughter cells. Checkpoint failure may cause cancer. Checkpoints play important roles in cell cycle arrest, decision of cell repair or death during arrest, and release from checkpoint arrest. Cell death induced by checkpoint release is the major mechanism of antitumor treatment. Sustained arrest inhibits cell death, resulting in genome instability and anticancer drug resistance.18
To demonstrate the involvement of HNF-1\(\beta\) in the abnormal cell cycle of ovarian CCA lines, HNF-1\(\beta\) knockdown cell lines were created to examine the effects of HNF-1\(\beta\) on the cell cycle and anticancer drug resistance. Bleomycin, a G2 agonist, sustained G2 arrest in the HNF-1\(\beta\) (+) group. A decrease in cell death rate was also observed in CCA cells expressing high levels of HNF-1\(\beta\). Of the proteins involved in checkpoint activation, CHK1 was sustainably phosphorylated. Checkpoints may be sustainably activated by HNF-1\(\beta\) in cancer cells through persistent CHK1 phosphorylation. This may cause genome instability, cancer, and anticancer drug resistance in ovarian CCA. It has been considered that endometriosis may be involved in the development of CCA. In endometriosis, repeated hemorrhage causes oxidative stress and generates free radicals. These free radicals should kill cells. However, HNF-1\(\beta\)-expressing cells may survive, and some of these cells may become cancerous.

The data presented here clearly show that overexpression of HNF-1\(\beta\) resulted in significant insensitivity to bleomycin, possibly through persistent activation of CHK1. More importantly, cell cycle progression and chemosensitivity of human CCA cells may be regulated by cellular HNF-1\(\beta\)-dependent CHK1 activation. This is the first study demonstrating cell cycle regulation and possibly chemosensitization by a CHK1 inhibitor in CCA. This suggests that cell cycle progression by inhibition of CHK1 can enhance tumor cell destruction by diverse genotoxic agents. Clinical administration of CHK1 inhibitors to patients with CCA may overcome many initial obstacles such as chemoresistance.

**FIGURE 3.** Activation of CHK1 is aberrantly sustained in HNF-1\(\beta\)-overexpressing cells after induction of G2 checkpoint. A, Western blot analysis of basal level of CHK1 in TU-OC-1-shHNF-1\(\beta\) and vector-control cells. B, Expression of phospho-CHK1 (serine296) in TU-OC-1-shHNF-1\(\beta\) and vector-control cells treated with 42 \(\mu\)M bleomycin. The time indicated was that after treatment. The corresponding \(\beta\)-actin levels are shown as a loading control.

**FIGURE 4.** CHK1 inhibitor leads to abrogation of G2 arrest and sensitizes HNF-1\(\beta\)(+) cells to DNA damaging agent induced cell death. A, Western blot analysis of phospho-CHK1 (Ser296) protein levels in TU-OC-1 cells treated with 50 nM CHK1 inhibitor 24 hours after the addition of 42 \(\mu\)M bleomycin. Cells were harvested at the indicated time points after treatment of CHK1 inhibitor. The corresponding \(\beta\)-actin levels are shown as a loading control. B, Cells were harvested and analyzed by flow cytometry at the indicated time points after treatment of CHK1 inhibitor.
This model predicts that tumors that overexpress HNF-1β would rely more heavily on the G2 checkpoint and be more sensitive to CHK1 inhibition than their normal cell counterparts. Because HNF-1β is overexpressed in most of ovarian CCA and renal CCA, both may offer a therapeutic window for selective sensitization of tumor cells to anticancer drugs by CHK1 inhibitors. Our hypothesis is supported by the findings that CHK1 inhibition preferentially sensitizes HCT116 p53−/− cells to gemcitabine20 and 5-fluorouracil.21 It may be premature to restrict CHK1 inhibitor use to HNF-1β-overexpressing tumors.

The mechanism by which phosphorylation of CHK1 is sustained is unclear. Our microarray data, reflecting HNF-1β-overexpressing tumors.