Role of $p21^{\text{waf1/cip1}}$ in effects of oxaliplatin in colorectal cancer cells

Taishi Hata, Hirofumi Yamamoto, Chew Yee Ngan, Minoru Koi, Akimitsu Takagi, Bazarragchaa Dadmansuren, Masayoshi Yasui, Yujiro Fujie, Takeshi Matsuzaki, Hiromichi Hemmi, Xundi Xu, Kotaro Kitani, Yosuke Seki, Ichiro Takemasa, Masataka Ikeda, Mitsugu Sekimoto, Nariaki Matsuura, and Morito Monden

1Department of Surgery and Clinical Oncology, Graduate School of Medicine and 2Department of Pathology, School of Allied Health Science, Faculty of Medicine, Osaka University, Osaka, Japan; 3Brunel Institute of Cancer Genetics and Pharmacogenomics, Department of Biological Sciences, Brunel University, Uxbridge, Middlesex, United Kingdom; 4Yakult Central Institute for Microbiological Research, Yaho, Kunitachi; and 5Department of Molecular Biology, Toho University Faculty of Medicine, Ohmori-Nishi, Ohta, Tokyo, Japan

Abstract

Clinical studies have shown that oxaliplatin, a novel platinum derivative, is a potent chemotherapeutic agent for colorectal cancer when combined with 5-fluorouracil and leucovorin. Although the toxic activity is based on covalent adducts between platinum and DNA, its actual biological behavior is mostly unknown. In an effort to explore the mechanism of tumor susceptibility to oxaliplatin, we examined the cytotoxic effects of oxaliplatin in colorectal cancer cell lines in reference to $p53$ gene status. Although $p53$ gene status did not clearly predict sensitivity to oxaliplatin, $p53$ wild-type cells including HCT116 were sensitive but HCT116 $p53^{-/-}$ were found to be resistant to oxaliplatin. Oxaliplatin caused strong $p21^{\text{waf1/cip1}}$ induction and $G_0$-$G_1$ arrest in $p53$ wild-type cells, whereas cisplatin did not induce $G_0$-$G_1$ arrest. Assays using $p53$ wild but $p21^{\text{waf1/cip1}}$ null HCT116 cells revealed that oxaliplatin did not show $G_0$-$G_1$ arrest and reduced growth-inhibitory effects, suggesting that $p21^{\text{waf1/cip1}}$ may be a key element in oxaliplatin-treated $p53$ wild-type cells. Although HCT116 is DNA mismatch repair–deficient, a mismatch repair–proficient HCT116 + ch3 cell line displayed similar responses with regard to $p21^{\text{waf1/cip1}}$-mediated growth inhibition and $G_0$-$G_1$ arrest. In p53 mutant cells, on the other hand, oxaliplatin caused an abrupt transition from $G_1$ to $S$ phase and eventually resulted in $G_2$-$M$ arrest. This abrupt entry into $S$ phase was associated with loss of the $p21^{\text{waf1/cip1}}$ protein via proteasome-mediated degradation. These findings suggest that $p21^{\text{waf1/cip1}}$ plays a role in oxaliplatin-mediated cell cycle and growth control in $p53$-dependent and -independent pathways. [Mol Cancer Ther 2005;4(10):1585–94]

Introduction

Cisplatin is a commonly used cancer agent that was discovered in 1968 and is effective in the clinical stage against carcinomas of the testis, ovary, and other malignancies (1, 2). By contrast, the efficiency of cisplatin is generally low in colorectal cancer, with <20% clinical responses when used alone or in combination with 5-fluorouracil or etoposide (3, 4). Oxaliplatin is a third-generation platinum coordination complex of the 1,2-diaminocyclohexane families, having been developed after cisplatin and carboplatin (5, 6). The mechanisms of action of cisplatin and oxaliplatin are theoretically similar and involve alkylation of DNA. Similar to cisplatin, oxaliplatin is inactivated by reaction with glutathione catalyzed by the glutathione-S-transferase enzyme. Oxaliplatin and cisplatin adducts are both repaired by the nucleotide excision repair system from which two enzymes, XPA and ERCC1, have been identified as being essential for the repair process (7, 8). Despite these similarities, the National Cancer Institute screening for susceptible cell lines showed distinct clustering of the oxaliplatin from other platinum compounds (9).

Compared with other classes of platinum derivatives, oxaliplatin seems particularly promising because it lacks the nephrotoxicity associated with cisplatin (10) and the myelosuppression associated with carboplatin treatments (11). Clinical studies have shown that oxaliplatin mono-therapy is effective against ovarian and colon cancer, melanoma, glioma, and non–Hodgkin’s lymphoma (12). Moreover, the combination of 5-fluorouracil, folinic acid, and oxaliplatin shows potent chemotherapy against metastatic colorectal cancer, with a response rate of about 50% (13).

The activity of platinum compounds is attributed to the formation of covalent adducts between platinum and some bases in the DNA of the cells, which leads to the inhibition of DNA synthesis (2, 14). Although oxaliplatin produces DNA crosslinks similar to those of cisplatin (15), differences still exist between the two agents as shown by the fact that cisplatin-resistant cells generally remain sensitive to...
oxaliplatin. The superb efficacy of oxaliplatin is considered to be due to the formation of bulkier platinum-DNA adducts and induction of a greater deformation of the DNA structure than cisplatin-DNA adducts (14, 16). However, the actual biological behavior of oxaliplatin is mostly unknown.

In an effort to explore the underlying mechanism associated with oxaliplatin, we examined the efficacy of oxaliplatin using six colorectal cancer cell lines with special attention to the status of the p53 gene. The p53 gene is a putative tumor suppressor that encodes a transcriptional factor for a set of genes involved in the regulation of cell cycle progression, DNA repair, and apoptosis, and thus it is often associated with chemosensitivity or irradiation-sensitivity (17–19). During the course of the study, we found oxaliplatin-mediated cell cycle--regulatory mechanisms in which cyclin-dependent kinase inhibitor p21waf1/cip1 may play a crucial role in p53-dependent and -independent pathways.

Materials and Methods

Reagents and Cell Lines

Oxaliplatin was obtained from Yakult Honsha Co., Ltd. (Tokyo, Japan). Cisplatin was purchased from Sigma-Aldrich, Co. (St. Louis, MO). The proteasome inhibitor, clasto-lactacystin β-lactone (Lactacystin) was purchased from Boston Biochem, Inc. (Cambridge, MA). HEK293 cells and six human colon cancer cell lines, HCT116, LoVo, DLD1, HT29, SW480, and CaCo2 were purchased from American Type Culture Collection (Rockville, MD) or the Japanese Cancer Research Resources Bank (Osaka, Japan). HCT116 p53+/+ cells or p21+/+ cells retain both the wild-type p53 gene and p21waf1/cip1 gene, whereas both alleles of the p53 gene or p21waf1/cip1 were deleted through homologous recombination in HCT116 p53−/− or HCT116 p21waf1/cip1−/− cells, respectively. These genetically impaired HCT116 cell lines and the parental cells with wild-type genes were generous gifts from Dr. Bert Vogelstein (Johns Hopkins University School of Medicine, Baltimore, MD). A subline complemented with chromosome 3 (HCT116+ch3) was obtained from M. Koi (Department of Biological Sciences, Brunel Institute of Cancer Genetics and Pharmacogenomics, Brunel University, United Kingdom). These chromosome 3-complemented cells were competent in DNA mismatch repair (20) and maintained in medium containing 0.4 mg/mL geneticin (Invitrogen, Carlsbad, CA). They were grown in DMEM supplemented with 10% fetal bovine serum, 100 units/mL penicillin, and 100 μg/mL streptomycin in 5% CO2 at 37°C.

Growth Assays

Cells were uniformly seeded (1 x 10⁵/well) into 12-well dishes in triplicate. Twenty-four hours later, the culture medium was removed and replaced with 2 mL of fresh medium containing antineoplastic reagent. After treatment with oxaliplatin for the indicated time intervals, cells were counted using a hemocytometer. All assays were repeated at least twice and no discrepant results were obtained between experiments. In the subsequent experiments, IC₅₀ concentration was used.

Cell Cycle Analysis

Flow cytometric analysis was done as described in our previous study (21). Briefly, after fixation in 70% cold ethanol, cell pellets were resuspended in 400 μL of 0.2 mg/mL propidium iodide containing 0.6% NP40 plus the same volume of 1 mg/mL RNase (Sigma-Aldrich) and then incubated in the dark at room temperature for 30 minutes. The cell suspension was then filtered through a 44-μm nylon mesh filter and data were acquired with a FACSort (Becton Dickinson Immunocytometry Systems, San Jose, CA). Analysis of the cell cycle was carried out using ModFIT software version 3.0 (Becton Dickinson Immunocytometry Systems). All assays were repeated and gave similar results.

Western Blot Analysis

Cells were washed twice with ice-cold PBS and collected with a rubber scraper. After centrifugation, the cell pellets were resuspended in lysis buffer [150 mmol/L NaCl, 50 mmol/L Tris aminomethane (pH 7.6), 20 mmol/L EDTA, 0.5% NP40, 1.0 mmol/L phenylmethylsulfonyl fluoride, 10 μg/mL aprotinin, and 10 μg/mL leupeptin]. After sonication, the extracts were clarified at 14,000 rpm for 25 minutes at 4°C, and the supernatant fraction was collected. Western blot analysis was done as described previously (22). The protein bands were detected using the Amersham enhanced chemiluminescence detection system (Amersham Biosciences Corp., Piscataway, NJ). Equal loading of the protein samples was confirmed by parallel Western blots for actin.

Antibodies

The following antibodies were used at appropriate concentrations as recommended by the manufacturer: anti-p21waf1/cip1 mouse monoclonal antibody (sc-187), anti-BAX mouse monoclonal antibody (sc-7480), anti-Gadd45 rabbit polyclonal antibody (sc-792; Santa Cruz Biotechnology, Inc., Santa Cruz, CA), anti-Rb mouse monoclonal antibody (G3-245; BD Biosciences, Santa Cruz, CA), anti-actin rabbit monoclonal antibody (A2066, Sigma-Aldrich), anti-cyclin B1 rabbit polyclonal antibody (Upstate Biotechnology, Inc., Lake Placid, NY), anti-CDC2 mouse monoclonal antibody (Cell Signaling Technology, Inc., Beverly, MA), anti-cyclin A1 mouse monoclonal antibody (Chemicon International, Temecula, CA), and anti-survivin rabbit polyclonal antibody (Novus Biologicals, Littleton, CO).

Immunocytochemistry

Cells were fixed in 10% buffered formalin for 10 minutes and 70% ethanol for 30 minutes. Immunostaining was done using the Vectastain avidin-biotin complex peroxidase kit (Vector Laboratories, Burlingame, CA). Slides were incubated with rabbit anticyclin B1 polyclonal antibody (Santa Cruz) at a dilution of 1:100 for 1 hour at room temperature. Nonimmunized mouse IgG (Vector Laboratories) or PBS alone was used as a substitute for the primary antibody in the negative controls.

Quantitative Real-time PCR for p21waf1/cip1 mRNA

Total cellular RNA was extracted using Trizol reagent (Life Technologies, Inc., Gaithersburg, MD). Complementary DNA was generated from 1 μg of RNA with avian

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myeloblastosis virus reverse transcriptase (Promega, Madison, WI). Quantitative real-time PCR was done using LightCycler (Idaho Technology, Inc., Salt Lake City, UT), as described in our previous study (23). Quantification data from each sample were analyzed using LightCycler analysis software. The transcription value of p21waf1/cip1 was determined by plotting on the standard curve constructed using HCT116 cells. The amount of each transcript was normalized according to that of porphobilinogen deaminase housekeeping gene quantified with the same sample. The primer sequences were as follows (23, 24):

- porphobilinogen deaminase sense, 5'-TGTCTGGTAACGGCAATGCGCCTGCAA3';
- porphobilinogen deaminase antisense, 5'-TCAATGTGTCGCCACACTGTCGGT3';
- p21waf1/cip1 sense, 5'-GTGGACCTGTCACTGTCTTG-3';
- p21waf1/cip1 antisense, 5'-CTTCCTCTTGAGGAAGACTAC-3';

**Adenoviral System for Production of Antisense Cyclin D1**

The adenoviral system for antisense cyclin D1 was constructed using AdEasy Adenoviral Vector System (25), which was a generous gift from Dr. Bert Vogelstein. Cyclin D1 complementary DNA [ref. (26); a generous gift from Dr. I. Bernard Weinstein (Herbert Irving Comprehensive Cancer Center, College of Physicians and Surgeons, Columbia University, New York, NY)] was subcloned into cytomegalovirus-driven shuttle vector in antisense orientation. Recombination with the adenoviral backbone vector was done in *Escherichia coli* BJ5183 cells by electroporation. Viral particles were amplified in HEK293 cells and then purified by CsCl banding.

**Statistical Analysis**

Data are expressed as mean ± SEM. Differences in cell growth were examined by the unpaired *t* test. *P* < 0.05 was considered statistically significant. Statistical analysis was done using StatView 5.0 (SAS Institute, Inc., Cary, NC).

**Results**

**Growth Inhibition of Colon Cancer Cell Lines**

Cell growth was assessed after 48 hours of exposure to 0 to 50 μmol/L of oxaliplatin. The IC50 values of each colon cancer cell line are shown in Table 1. A 72 h treatment of oxaliplatin at IC50 concentration significantly suppressed growth of HCT116 p53 wild-type cells compared with the control culture (*P* = 0.0034), whereas HCT116 p53 mutant cells were resistant to such treatment. A repeat experiment yielded similar results. B, expression of p53 downstream genes. p21waf1/cip1, Bax, and Gadd45, were examined following 48 h of treatment with oxaliplatin at IC50 concentration. pRb, the substrate of cyclin-dependent kinase, was also examined. Blots for actin served as loading control. HCT116, p53 wild-type; SW480 and DLD1, p53 mutant type.

**Figure 1.** A, effect of oxaliplatin (L-OHP) on growth of HCT116 p53+/+ and HCT116 p53−/− cells. A 72 h treatment of oxaliplatin at IC50 concentration significantly suppressed growth of HCT116 p53−/− cells compared with the control culture (*P* = 0.0034), whereas HCT116 p53−/− cells were resistant to such treatment. A repeat experiment yielded similar results. B, expression of p53 downstream genes. p21waf1/cip1, Bax, and Gadd45, were examined following 48 h of treatment with oxaliplatin at IC50 concentration. pRb, the substrate of cyclin-dependent kinase, was also examined. Blots for actin served as loading control. HCT116, p53 wild-type; SW480 and DLD1, p53 mutant type. C, p21waf1/cip1 expression in various colorectal cancer cell lines. Marked induction of p21waf1/cip1 was noted in p53 wild-type cell lines, HCT116 and LoVo. However, HCT116 p53−/− cells did not induce p21waf1/cip1.

<table>
<thead>
<tr>
<th>Cell line</th>
<th>p53 status</th>
<th>Oxaliplatin IC50(μmol/L)</th>
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<tbody>
<tr>
<td>HCT116</td>
<td>wild-type</td>
<td>0.4 ± 0.1</td>
</tr>
<tr>
<td>LoVo</td>
<td>wild-type</td>
<td>2.5 ± 0.3</td>
</tr>
<tr>
<td>DLD1</td>
<td>mutant</td>
<td>9.1 ± 0.3</td>
</tr>
<tr>
<td>HT29</td>
<td>mutant</td>
<td>4.0 ± 0.4</td>
</tr>
<tr>
<td>CaCo2</td>
<td>mutant</td>
<td>1.0 ± 0.2</td>
</tr>
<tr>
<td>SW480</td>
<td>mutant</td>
<td>2.1 ± 0.3</td>
</tr>
</tbody>
</table>

*Colon cancer cell lines were treated with various concentrations of oxaliplatin for 48 hours and the IC50 value was calculated.*
Figure 2. Cell cycle analysis. Twelve to 36 h of exposure to oxaliplatin (L-OHP) increased the G0-G1 phase fraction in p53 wild-type cell lines (HCT116 and LoVo), and decreased the S phase fraction. Twelve to 24 h of exposure to oxaliplatin rapidly decreased the G0-G1 phase fraction in other p53 mutant cell types (SW480, CaCo2, DLD1, and HT29), increased the S phase fraction, and eventually caused arrest at G2-M phase. IC50 concentration for each cell line was applied.

*, statistical significance ($P < 0.05$).
Effect of Oxaliplatin on HCT116 p53+/+ and HCT116 p53−/− Cells

Treatment with oxaliplatin at IC₅₀ concentration significantly suppressed cell growth of HCT116 p53+/+ compared with control culture devoid of oxaliplatin (P = 0.0034). In contrast, HCT116 p53−/− cells were resistant to oxaliplatin (Fig. 1A).

Expression of Specific Genes Acting Downstream of p53

We then examined the effect of oxaliplatin treatment on the expression of p53 downstream genes such as p21⁰⁰⁰₁/cip¹, Bax, and Gadd45 (Fig. 1B). We also examined the effect of the same treatment on pRb (retinoblastoma gene protein), the substrate of cyclin-dependent kinase. Oxaliplatin treatment of HCT116 cells increased p21⁰⁰⁰₁/cip¹ protein level, and decreased Gadd45 level. In concordance with the p21⁰⁰⁰₁/cip¹ induction, the underphosphorylated form of pRb (active form as cell cycle negative regulator acting at G1-S transition) became dominant. Oxaliplatin treatment of SW480 and DLD1, in which the p53 status is mutant, resulted in up-regulation of Bax in SW480 only. The same treatment in all colorectal cancer cell lines caused a marked p21⁰⁰⁰₁/cip¹ induction in wild-type p53 cell types HCT116 and LoVo, but no remarkable induction was noted in mutant p53 cell types (Fig. 1C). Experiments using HCT116 p53−/− cells showed the lack of p21⁰⁰⁰₁/cip¹ induction.

Cell Cycle Analysis

The cell cycle was analyzed in cultures with and without oxaliplatin. In accordance with p53 gene status, two distinct patterns of cell cycle progression were noted. Thus, in p53 wild-type cell lines, HCT116 and LoVo, oxaliplatin treatment significantly increased G0-G1 phase fraction, and decreased the S phase fraction. In the other p53 mutant cell types, the G0-G1 phase fraction significantly decreased rapidly and the S phase fraction in turn increased with oxaliplatin, followed ultimately by G2-M arrest (Fig. 2).

Cell Cycle and Growth of HCT116 p21⁰⁰⁰₁/cip¹+/+ and HCT116 p21⁰⁰⁰₁/cip¹−/−

To further investigate whether the G1 delay in p53 wild-type cells is the direct effect of p21⁰⁰⁰₁/cip¹ induction, cell cycle analysis was done using HCT116 p21⁰⁰⁰₁/cip¹+/+ and HCT116 p21⁰⁰⁰₁/cip¹−/− cells. Both cell types retained...
normal p53 gene. Oxaliplatin at IC50 concentration (0.4 μmol/L) significantly induced G0-G1 accumulation in HCT116 p21waf1/cip1+/+, but not in HCT116 p21waf1/cip1−/− cells (Fig. 3A). Similarly, using oxaliplatin, HCT116 p53+/+ cells showed G1 arrest whereas HCT116 p53−/− cells did not (data not shown). When cisplatin was administered for comparison at its IC50 concentration (1.0 μmol/L), HCT116 p21waf1/cip1+/+ culture showed no change in G0-G1 or S fraction compared with the control cultures, and accumulation of the G2-M cell fraction. In HCT116 p21waf1/cip1−/−, cisplatin resulted in distribution of cell population similar to that in HCT116 p21waf1/cip1+/+ (Fig. 3A).

In growth-inhibitory assays, oxaliplatin largely inhibited the growth of HCT116 p21waf1/cip1+/+, but inhibitory effects were significantly smaller in HCT116 p21waf1/cip1−/−. These differences were significant (P < 0.001; Fig. 3B). On the other hand, the growth-inhibitory effects of cisplatin were not affected by p21waf1/cip1 gene status (Fig. 3B).

**Restoration of the DNA Mismatch Repair System in HCT116**

Because HCT116 is known as a DNA mismatch repair–deficient cell line (20), we examined whether the above findings of p21waf1/cip1 induction and G0-G1 arrest with oxaliplatin treatment are independent of mismatch repair deficiency or not. Comparative experiments using the mismatch repair–deficient HCT116 and mismatch repair–proficient HCT116+ch3 revealed that treatment with oxaliplatin at IC50 induced p21waf1/cip1 expression in both cell lines (Fig. 4A) and displayed similar growth inhibition.

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**Figure 4.** Comparative experiments using mismatch repair–deficient HCT116 and mismatch repair–proficient HCT116+ch3. A, oxaliplatin (L-OHP) treatment at IC50 induced prominent p21waf1/cip1 expression at 48 h in both cell lines. The actin blot served as a loading control. B, similar growth-inhibitory effects were observed in both cell lines following 72-h treatment with oxaliplatin. No significant difference in growth inhibition rate was found. A 48-h treatment also showed similar growth suppression in both cell lines (data not shown). C, cell cycle analysis indicated that oxaliplatin treatment increased G0-G1 phase fraction and decreased the S phase fraction at 24 and 36 h. Significant differences (*, P < 0.05 and **, P < 0.01) were obtained from two independent experiments.
Cell cycle analysis indicated that oxaliplatin treatment increased G0-G1 phase fraction and decreased the S phase fraction at 24 and 36 hours in both cell lines (Fig. 4C). A repeat experiment gave reproducible results and provided statistical significance.

### p21waf1/cip1 Protein and mRNA Expression in Oxaliplatin-Treated p53 Mutant Cells

In flow cytometric analysis, p53 mutant cells commonly showed abrupt entry from G0-G1 into S phase when treated with oxaliplatin. Time course experiments showed that treatment of SW480 and DLD1 cell cultures with oxaliplatin reduced p21waf1/cip1 protein expression as early as 24 hours and then decreased progressively (Fig. 5A). The addition of lactacystin, a proteasome inhibitor, to oxaliplatin-treated cultures at the indicated concentrations, increased the level of p21waf1/cip1 protein (Fig. 5A). In contrast, oxaliplatin treatment increased the expression of p21waf1/cip1 mRNA (Fig. 5B).

#### Possible Role of Abrupt Entry of p53 Mutant Cells from G0-G1 to S Phase

To explore the role of abrupt entry of oxaliplatin-treated p53 mutant cells from G0-G1 to S phase, we infected SW480 and DLD1 cells with antisense cyclin D1 and oxaliplatin. Western blot analysis indicated a 75% reduction in cyclin D1 protein, compared with mock-infected control; growth-inhibitory effects of oxaliplatin (IC50). When oxaliplatin was applied 2 h after infection and cells incubated for 2 d, cell growth of cultures infected with adenovirus antisense cyclin D1 was less inhibited than that of mock-infected control cultures (P = 0.036).

#### CDC2 and survivin Expression in p53 Mutant Cells

Expressions of CDC2 and survivin protein were examined following 48 h of treatment with oxaliplatin at IC50 concentration. Oxaliplatin generally decreased the levels of these proteins. Actin served as a loading control.

#### Immunostaining of Cyclin B1

HT29 cells with and without treatment of oxaliplatin. A 48-h treatment of oxaliplatin at IC50 generally decreased the cell population with nuclear cyclin B1. Cytoplasmic staining of cyclin B1 was evident with oxaliplatin treatment. Magnification, ×100.
adeno virus to induce antisense cyclin D1 mRNA into p53 mutant DLD1 cells. Western blotting indicated that there was an ~75% reduction in cyclin D1 protein, compared with mock-infected cultures (Fig. 6A). Administration of oxaliplatin results in less inhibition of cell growth of antisense cyclin D1-infected cultures than that of mock control–infected cultures (P = 0.036; Fig. 6A). This experiment was repeated thrice and similar results were obtained.

**Mechanism of G2-M Arrest in p53 Mutant Cells**

Because p53 mutant cells showed G2-M arrest, we examined the expression of CDC2, a crucial positive regulator acting at the G2-M phase. Treatment of oxaliplatin exclusively decreased the expression of the CDC2 protein in p53 mutant cell lines (Fig. 6B). When we examined the expression of the cyclin B1 protein, the catalytic partner of CDC2, the expression level of cyclin B1 was not affected with oxaliplatin treatment (data not shown). On the other hand, oxaliplatin exclusively decreased the expression of survivin, which is a modulator for both G2-M transition and antiapoptosis (Fig. 6B; ref. 28). Finally, we examined intracellular localization of cyclin B1 because nuclear translocation from the cytoplasm is important in G2-M transition (29). Immunostaining of cyclin B1 revealed that oxaliplatin generally decreased cell population with nuclear cyclin B1 (Fig. 6C; Table 2). Significant decreases in nuclear cyclin B1 were noted in SW480, HT29, and DLD1 (P = 0.014, 0.011 and < 0.0001, respectively).

**Discussion**

Colorectal cancer is considered to be relatively resistant to chemotherapy, compared with other types of human malignancies such as ovarian cancer and esophageal cancer (30). In the last decade, however, chemotherapy against colorectal cancer gradually achieved improved clinical outcomes by using the combination of two or three chemotherapeutic agents (31). Using 5-fluorouracil as a key agent, combination with levamisole (32), or leucovorin (33, 34) provided clinical efficacy (18% and 12–33%, respectively). The combined regimen of 5-fluorouracil, leucovorin, and CPT11 was effective in 35% to 39% (35, 36). In 2000, another attractive regimen (FOLFOX) was reported using oxaliplatin in combination with levamisole (32), or leucovorin and CPT11, providing a higher clinical efficacy of 51% (13). In addition to the above clinical (and preclinical) synergy in combination with S phase inhibitors, there are elements specific to oxaliplatin. These include exquisite potency in sensitive lines, killing with a fraction of the platinum-DNA adducts (2, 14–16), and no resistance due to mismatch repair deficiency (37). DNA repair–related resistance to cisplatin could be due to an increase in nucleotide excision repair or to a defect in DNA mismatch repair, resulting from replicative bypass of platinum adducts (38). The same defects in mismatch repair or replicative bypass, however, did not confer resistance to oxaliplatin (39).

The present study suggests that p21waf1/cip1 might play a role in exerting the activity of oxaliplatin in p53-dependent and p53-independent pathways (Fig. 7). In a p53-dependent pathway, oxaliplatin enhanced the expression of p21waf1/cip1 protein, which may be responsible for the growth-inhibitory activity by delaying the transition from G0-G1 to S phase. In HCT116 cells, the hyperphosphorylated form of pRb was reduced by oxaliplatin, probably because induction of p21waf1/cip1 inhibited cyclin-dependent kinase activity. This hypothesis is further strengthened by the results of experiments using genetically impaired HCT116 cells. p53 null HCT116 cells lost both the growth-inhibitory effect and G1-S arrest pattern of the cell cycle, indicating that the p53 gene seems to play a crucial role in mediating the effect of oxaliplatin in p53 wild-type cells. These findings are consistent with the results of Manic et al. who reported that oxaliplatin is more resistant to cell types of mutant type p53 in osteosarcoma and carcinomas of the ovary and uterus (40), and is also in agreement with a recent report which showed that inactivation of p53 or Bax resulted in increased resistance to oxaliplatin (41). In the present study, we found that HCT116 p53−/− cells did not induce p21waf1/cip1, suggesting that the lack of a functional p53 protein could lead to reduced p21 protein levels in response to oxaliplatin exposure. Moreover, p21waf1/cip1 null but p53 normal HCT116 cells offered a reason for p53-dependent growth inhibition because these cells lost not only G0-G1 arrest pattern but also the growth-inhibitory effect by oxaliplatin. Furthermore, using the

**Table 2. Nuclear translocation of cyclin B1**

<table>
<thead>
<tr>
<th></th>
<th>Oxaliplatin (−), %</th>
<th>Oxaliplatin (+), %*</th>
<th>P</th>
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<tbody>
<tr>
<td>SW480</td>
<td>53.8 ± 0.6</td>
<td>38.2 ± 2.6</td>
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<tr>
<td>HT29</td>
<td>64.4 ± 3.2</td>
<td>50.9 ± 5.8</td>
<td>0.011</td>
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<tr>
<td>DLD1</td>
<td>71.6 ± 4.2</td>
<td>53.2 ± 2.0</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Caco2</td>
<td>40.2 ± 0.3</td>
<td>30.9 ± 4.4</td>
<td>0.095</td>
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</tbody>
</table>

*Colon cancer cell lines were treated with oxaliplatin at IC50 for 48 hours and stained with cyclin B1 antibody."
mismatch repair–proficient HCT116 + ch3, we confirmed that the mismatch repair–deficient nature of HCT116 was independent of p21-mediated growth inhibition and G0-G1 arrest. Taken together, these findings suggest that p21\textsuperscript{waft/cip1} might be the responsible element.

Another study showed that oxaliplatin in HCT116 cells resulted in a minor degree of apoptosis via p53- and Bax-dependent pathways and the major response was a mixed G1 and G2 growth arrest at 10- to 20-fold higher concentrations (4 or 8 \mu mol/L) that of that used in the present study (IC\textsubscript{50} 0.4 \mu mol/L; ref. 42). Although our data showed that G1 arrest was a major characteristic of p53 wild-type cells, when the p21 gene of HCT116 was inactivated, G1 accumulation disappeared and instead the G2-M population became evident. Moreover, Arango et al. (41) showed that oxaliplatin induced G2-M arrest in HCT116 at higher concentrations (5–50 \mu mol/L), suggesting that induction of G2-M arrest in HCT116 might be dose-dependent.

On the other hand, it is known that p21\textsuperscript{waft/cip1} can be induced by p53-independent stimuli such as IFN-\alpha, IFN-\gamma (43, 44), and transforming growth factor-\beta (45). Although the level of p21\textsuperscript{waft/cip1} expression was lower in p53 mutant cells than in p53 wild-type cells, a modest level of p21\textsuperscript{waft/cip1} expression was still observed in SW480 and DLD1. We found that oxaliplatin decreased p21\textsuperscript{waft/cip1} expression in these cells, which was likely to facilitate abrupt exit from G0-G1 and entry into S phase. It seems that a transient increase and decline in S phase represents the population of cells that continue to progress through the cell cycle and later accumulate at G2-M. Comparison of p21\textsuperscript{waft/cip1} expression at protein and mRNA levels revealed that proteasome-mediated degradation of the p21\textsuperscript{waft/cip1} protein could be operational in oxaliplatin treatment. Proteasome-mediated degradation of p21\textsuperscript{waft/cip1} is reported in either ubiquitous or nonubiquitous pathways, depending on the cell context (46, 47).

To address the functional significance of abrupt entry into S phase in p53 mutant cells, we did an additional experiment to block entry from G1 into S phase. For this purpose, we suppressed cyclin D1, a putative G1 cyclin acting in G1-S transition, using the adenovirus system. Because DLD1 requires only a small titer of viral particles (20–30 multiples of infection), whereas SW480 required a higher titer of >200 multiples of infection, which is likely to be toxic in combination with chemotherapeutic agents, we employed DLD1 cells in this experiment. Although we had anticipated that the blockade of G1 to S transition would be more efficient by carrying out concurrent arrests at G0-G1 and G2-M phases, antisense cyclin D1 weakened the efficacy of oxaliplatin rather than strengthening it. Based on these results, we hypothesized that the abrupt entry into S phase might be an indispensable procedure to fulfill the maximum efficacy of oxaliplatin, possibly as an important pre-stage to G2-M arrest. We found that oxaliplatin down-regulated CDC2 protein and decreased nuclear translocation of cyclin B1. This could be at least one mechanism. In addition, we previously found that oxaliplatin reduced levels of antipapoptotic survivin in some tumor cells. In this study, we extended the findings by showing that oxaliplatin decreased survivin in all p53 mutant cell lines tested. It is reported that survivin localizes to centrosomes, mitotic-spindle microtubules and midbodies, mainly to carry out controlled distribution of chromatin and maintain the mitotic process in order (48). Therefore, our findings of oxaliplatin-mediated down-regulation of survivin may partially account for the inhibition of cell division at mitosis.

Although p53 status alone was not a predictor of chemosensitivity to oxaliplatin, it is apparent from this study that wild-type p53 in tumor tissues could be at least one factor in predicting drug efficacy. Because ~50% of colorectal cancers retain wild-type p53 (49), the present study warrants further investigation; whether the p53 wild-type group is generally susceptible to oxaliplatin in a large-scale clinical study needs to be done to refine tailor-made treatment.

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