Effect of 5-Fluorouracil on Cell Cycle Regulatory Proteins in Human Colon Cancer Cell Line

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We investigated the effects of 5-fluorouracil (5-FU) on cell cycle-regulating proteins in RPMI 4788 cells. 5-FU inhibited cell growth dose-dependently and this growth inhibition was accompanied with cell cycle accumulation in early S phase and increased expression of cyclin A. When cells were released from short-term treatment (3 or 24 h) with 5-FU, the cell cycle started to progress again and cyclin A protein levels decreased. Cyclin A-associated kinase activity assay showed that cyclin A-cyclin-dependent kinase (Cdk) 2 kinase activity was altered by 5-FU treatment concomitantly with the changes in cell cycle state seen in flow cytometric analysis. Furthermore, the elevation of cyclin A protein level by 5-FU treatment was observed in three other human cancer cell lines, DLD-1, H226Br and T.Tn. These results suggest that cyclin A protein levels in cancer cells are increased by 5-FU, and the cyclin A function and degradation mechanism remain normal.

Key words: 5-FU — Cyclin A — Cell cycle — Exposure time

5-Fluorouracil (5-FU) has been used clinically for over 30 years and it exerts its effect on proliferating cells by interfering with DNA synthesis. Many aspects of the cellular and molecular pharmacology of 5-FU have been investigated in vitro, as well as in vivo. In some studies, sensitivity of growing cells to 5-FU was observed more or less in all cell cycle stages,1, 2) while other studies have found that 5-FU blocks the cell cycle at the G1 and S phases.3, 4) The mechanisms by which 5-FU modulates cell cycle regulation have not been clarified.

Recently, considerable progress has been made in identifying proteins whose expression and activity regulate the cell cycle. The progression through the cell cycle is thought to be driven by sequential activation of cyclin-dependent kinases (Cdks) through binding to specific cyclins.5-7) Cyclin A is a cell cycle regulating protein that binds to Cdk2 or Cdc2 proteins and plays an important role in regulating the S phase transition.8)

In this paper, we investigated the effect of 5-FU on cell cycle progression from the viewpoint of the cell cycle regulatory proteins, using asynchronous cells.

MATERIALS AND METHODS

Cell culture and experimental group RPMI 4788 cells derived from human colon cancer (supplied by Roswell Park Memorial Institute, Buffalo, NY) were maintained in RPMI 1640 medium supplemented with 10% fetal calf serum (FCS). Cells were seeded in 75-cm² tissue culture bottles at 5×10⁶ cells/bottle, and cultured at 37°C in a 5% CO₂ incubator. After 72 h preculture, culture medium was changed to RPMI 1640 medium containing 10% FCS with or without 5-FU (0.1 to 100 µM) for in vitro experiments. 5-FU was purchased from Kyowa Hakko Kogyo Co., Ltd., Tokyo. In the continuous exposure experiment, cells were harvested at the time point of 6, 12, 24 or 48 h for the following experiments. In the short-term exposure experiment, cells were exposed for 3 or 24 h to 5-FU, followed by culture without 5-FU for 24, 48 or 72 h. Viable cells were washed three times with ice-cold phosphate-buffered saline (PBS), trypsinized and counted by the dye-uptake method. Other human cancer cell lines, DLD-1 (colon cancer), H226Br (lung squamous cell cancer), and T.Tn (esophagus cancer), were also used in some experiments.

Flow cytometric analysis At the indicated times, RPMI 4788 cells treated with or without 5-FU were trypsinized and washed twice with ice-cold PBS containing 1% FCS. Cell pellets were suspended in 0.5 ml of PBS plus NaN₃ (>2×10⁶ cells/ml) and the same volume of the buffer (0.1% Triton-X100, 1 mg/ml RNase A in PBS) and passed through the mesh. The same volume of propidium iodide (PI) solution (0.1 mg/ml PI in PBS) was added to the sample and the mixture was incubated for 15 min. Flow cytometric analysis was performed using a FACScalor cytometer (Becton Dickinson, Mountain View, CA).

Immunoblotting Immunoblotting of protein extracts were performed as previously described.10) At the indicated times, viable cells were harvested by scraping and

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were directly solubilized in sodium dodecyl sulfate (SDS) sample buffer (125 mM Tris pH 6.8, 4% SDS, 20% glycerol). An equal amount of total proteins obtained from equivalent numbers of viable cells (approximately 2×10^5 cells/lane) was separated on 12% SDS polyacrylamide gel and transferred to a nitrocellulose filter. The filter was blocked for 1 h at room temperature in tris-buffered saline (TBS)/milk buffer (50 mM Tris pH 7.4, 100 mM NaCl, 2% skimmed milk), then incubated overnight at room temperature with primary antibodies in TBS/milk buffer. Anti-cyclin A polyclonal antibodies [rabbit immunoglobulin G (IgG)], anti-Cdk2 polyclonal antibodies (rabbit IgG) and anti-Cdc2 polyclonal antibodies (rabbit IgG) were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA) and used as primary antibodies. Proteins were visualized using an alkaline phosphatase-conjugated goat anti-rabbit IgG as the secondary probe.

**Immunoprecipitation and histone H1 kinase assay**

Equivalent numbers of viable cells (3×10^6 cells) were lysed in lysis buffer (0.2% Triton-X100, 20 mM Tris pH 8.0, 100 mM NaCl, 1 mM 2-mercaptoethanol (2-ME), 3 mM phenylmethylsulfonyl fluoride (PMSF)) and total extracts were immunoprecipitated by anti-cyclin A, anti-Cdk2, or anti-Cdc2 antibodies and protein A Sepharose beads. The kinase activity of precipitates was assayed by using histone H1 as a substrate, as previously described. Briefly, immunoprecipitates were rinsed with high salt (0.2% Triton-X100, 20 mM Tris pH 8.0, 300 mM NaCl, 1 mM 2-ME, 3 mM PMSF) and low salt (0.1% Triton-X100, 10 mM Tris pH 8.0, 10 mM NaCl, 1 mM 2-ME, 3 mM PMSF) washing buffers. Kinase reaction mixture (20 mM Tris pH 8.0, 10 mM MgCl_2, 1 mM 2-ME, 3 mM PMSF) was added to immunoprecipitates together with [γ-32P]ATP (2.5 µCi/sample), and histone H1 (1 µg/sample) and the mixture was incubated for 1 h at 25°C. The reaction was terminated by adding SDS sample buffer and proteins were separated on a 12% SDS polyacrylamide gel. Bands were detected by autoradiography.

**RESULTS**

**Time course of tumor cell growth**

Dose-dependent inhibition of tumor cell growth by the 5-FU treatment was observed. At the concentration of 0.1 µM, 5-FU did not show growth inhibition compared with the control (data not shown). In the continuous exposure experiment, the values of percent growth inhibition by 1, 10 and 100 µM 5-FU treatment were 16, 46 and 56% at 24 h, and 32, 74 and 77% at 48 h, respectively (Fig. 1A). In the 3-h treatment group, inhibition of tumor cell growth was seen only at 100 µM 5-FU, not at 1 or 10 µM (Fig. 1B). In the 24-h treatment group, cell growth was not affected at the concentration of 1 µM 5-FU, while 10 or 100 µM 5-FU was inhibitory (Fig. 1C).

**Flow cytometric analysis**

The continuous exposure experiment showed that 5-FU affected the cell cycle time- and dose-dependently. In the control and 0.1 µM treatment groups, the cell cycle patterns were the same (data not shown).

![Fig. 1. Cell growth curves of 5-FU-treated RPMI 4788 cells. Cells were cultured in culture medium for 72 h and then treated without 5-FU (control ○), with 1 µM 5-FU (1 µM □), with 10 µM 5-FU (10 µM △) or with 100 µM 5-FU (100 µM ×) for 24 or 48 h. After short-term exposure (3 or 24 h), cells were washed and cultured without 5-FU for the time (h) indicated. Cells were trypsinized and counted by the dye-uptake method. A, continuous exposure; B, short-term exposure (3 h); C, short-term exposure (24 h).](image-url)
Fig. 2. DNA histograms of 5-FU-treated RPMI 4788 cells. Cells were cultured in culture medium for 72 h and then treated without 5-FU (control), with 1 µM 5-FU (1 µM), with 10 µM 5-FU (10 µM) or with 100 µM 5-FU (100 µM). Cells were analyzed by FCM at 6, 12, 24 and 48 h in the continuous exposure experiment (A) or at 24, 48 or 72 h in the 3- or 24-h exposure experiment (B, C).
Effects of 5-FU on cyclin A, Cdk2 and Cdc2 protein levels

We examined the protein levels of cyclin A, Cdk2 and Cdc2 after treatment with 5-FU. At 6 h after treatment, cyclin A protein levels showed no remarkable change. At 12, 24 or 48 h after treatment, however, cyclin A protein levels gradually increased in a dose-dependent manner. Cdk2 and Cdc2 protein levels showed no significant differences among the experimental groups (Fig. 3A). Twenty-four hours after wash-out of 3-h 5-FU treatment, cyclin A protein levels were increased only in the case of 100 \( \mu \)M 5-FU treatment, and thereafter decreased gradually. In the 24-h exposure experiment, 10 and 100 \( \mu \)M 5-FU treatment caused increases in cyclin A protein level compared with the control, although the Cdk2 protein level showed no difference (Fig. 3B). We also examined cyclin A protein levels in three other human cancer cell lines. All three cell lines were susceptible to 5-FU and the expression of cyclin A were analyzed by immunoblotting (Table I). Analysis was performed after 24 h (RPMI 4788, DLD-1) or 48-h treatment (H226Br, T.Tn) with 5-FU and data are shown as the ratio to the control. Cyclin A protein levels were increased by 5-FU treatment in all cell lines examined.

**Histone H1 kinase assay**

Six-hour treatment with 5-FU caused no remarkable change in the histone H1 kinase activity of the cyclin A immunoprecipitate in the 0.1 and 1 \( \mu \)M groups compared with the control group, but the 1 and 10 \( \mu \)M 5-FU group showed increased levels of histone H1 kinase activity. In the case of 12-h treatment, the 0.1 \( \mu \)M group showed the same histone H1 kinase activity as that of the control group, but the 1 and 10 \( \mu \)M groups showed increased histone H1 kinase activities. After wash-out following 3-h or 24-h 5-FU treatment, the histone H1 kinase activity of the cyclin A immunoprecipitate paralleled the cyclin A protein level (Fig. 4B).

**DISCUSSION**

5-FU is a cell cycle-phase-dependent anticancer drug, and its effects on cells are therefore dependent on both the concentration and exposure time.\(^{12}\) The cytotoxicity of 5-FU is due to inhibition of DNA synthesis mediated by the ability of 5-FU to inhibit the enzyme thymidylate synthetase. However, it is becoming apparent that 5-FU incorporation into RNA also has an important role in its cytotoxicity.\(^{13}\) Generally, DNA-directed actions are manifested in the S phase and RNA-directed actions in the G1 phase.\(^{3}\) Since the DNA-directed actions of 5-FU are expressed in the S phase specifically, 5-FU exhibits a potent action if treatment is continued for a long time, even at a low dose.\(^{14,15}\) In a previous report, flow cytometric analysis of the cell-killing action of 5-FU in a human colon cancer cell line showed that most of the treated cells accumulated in the S phase.\(^{16}\) In another flow cytometric analysis in two human colon cancer cell lines, RNA- or DNA-directed cytotoxicity due to 5-FU exposure resulted nearly complete disappearance of S phase cells or accumulation of cells at the G1-S border, respectively.\(^{17}\) In our experiment, 5-FU caused dose-dependent growth inhibition of RPMI 4788 cells. According to the flow cytometric analysis, this growth inhibition was accompanied with cell cycle alteration. In the short-term exposure experiment, 5-FU inhibited the cell growth dose-dependently and release from 5-FU exposure resulted in the recovery of cell cycle progression. Twenty-four hours of 5-FU exposure produced more effective growth inhibition than 3 h, suggesting that the effect of 5-FU on cell growth is dependent on both the concentration and exposure time.

To investigate the effect of 5-FU on cell cycle-regulating proteins, mainly those which participate in the S phase transition, we examined the amount and function of cell cycle-related protein by western blotting and kinase activity measurement. Cyclin A protein levels of untreated cells

| Cell line | Control | 5-FU 1 \( \mu \)M | 5-FU 10 \( \mu \)M |
|-----------|---------|-----------------|-----------------|
| RPMI 4788 | 1.00    | 3.71            | 4.47            |
| DLD-1     | 1.00    | 1.56            | 6.26            |
| H226Br    | 1.00    | 2.39            | 4.74            |
| T.Tn      | 1.00    | 3.08            | 3.46            |
Fig. 3. Effect of 5-FU on the expression of cell cycle regulatory proteins in RPMI 4788 cells. Cells were harvested and analyzed by immunoblot analysis at 6, 12, 24 and 48 h after the start of incubation with or without 5-FU (A), or at 24, 48 or 72 h after 3 or 24 h 5-FU exposure (B). Equal volumes of whole cell extracts obtained from equivalent numbers of viable cells (1×10⁵ cells/lane) which contained approximately 100 µg of protein were separated and electrophoretically blotted. Proteins were identified with anti-cyclin A, anti-Cdk2 and anti-Cdc2 antibody.
Fig. 4. Effect of 5-FU on the kinase activity of cell cycle regulatory proteins in RPMI 4788 cells. Equal volumes of whole-cell extracts obtained from equivalent numbers of viable cells (1×10^6 cells) were immunoprecipitated with anti-cyclin A, anti-Cdk2 and anti-Cdc2 antibody, and the histone H1 kinase activity of immunoprecipitates was analyzed as described in “Materials and Methods.” A, continuous exposure; B, short-term exposure.
increase from G1 to S phase and decrease in the G2M phase.\(^{18}\) In a recent report, expression of cyclin A was claimed to be an early event in the S phase and the maximal rate of accumulation of cyclin A was achieved during the first hour of progression through the S phase.\(^{19}\) Our experiments revealed that 5-FU increased both the expression of cyclin A protein and its associated kinase activity \textit{in vitro}. According to our experiment, cyclin A protein levels were increased in a dose-dependent manner by continuous exposure to 5-FU, and when the cell cycle progressed from S phase to G2 phase, cyclin A protein levels gradually decreased. The elevation of cyclin A protein level by 5-FU treatment was also observed in three other human cancer cell lines indicating that this result could be general. We also observed that cyclin A-associated and Cdk2-associated kinase activities were increased by 5-FU even though the amount of Cdk2 protein did not vary during the experiment. In contrast, 5-FU treatment has no effect on the activity of Cdc2 immunoprecipitates. The cyclin A-Cdk2 complex is considered to be involved in DNA replication in higher eukaryotes.\(^{20}\) We found that the kinase activity was correlated with the results of flow cytometry, suggesting that the increased cyclin A activity was associated with Cdk2 and that the function of the cyclin A-Cdk2 complex was maintained during 5-FU treatment. When 5-FU was removed, the cell cycle started to progress again and moved to the G2M phase. At that time, cyclin A-associated kinase activities were still increased, but Cdk2-associated kinase activities were decreased. Presumably this cyclin A kinase activity in the G2M phase might be related to Cdc2 because the cyclin A-Cdc2 complex works in the G2M phase. Thus, cyclin A protein appears to maintain its function even after 5-FU treatment, and cyclin A protein level and kinase activity might be used as an index of 5-FU effect.

Generally, when we use 5-FU 500 mg/day continuously in the clinic, the mean values of 5-FU serum concentrations range from 1 to 2 \(\mu M\).\(^{21}\) According to our \textit{in vitro} experiments, cells accumulate in the S phase at this concentration, but after release, the cell cycle started to progress because cancer cells free from 5-FU have a normal cyclin A or cyclin A-complex function. Therefore in order to kill cancer cells effectively, better biochemical modulation or a cell cycle-specific apoptosis inducer should be combined with 5-FU treatment.

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