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ORIGINAL ARTICLE

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Mechanism of vinorelbine-induced radiosensitization of human small cell lung cancer cells

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Abstract Vinorelbine (Navelbine, KW-2307), a semisynthetic vinca alkaloid, is a potent inhibitor of mitotic microtubule polymerization. The aims of this study were to demonstrate vinorelbine-induced radiosensitization of human small cell lung cancer (SCLC) SBC-3 cells and to elucidate the mechanisms of radiosensitization. A clonogenic assay demonstrated that SBC-3 cells were sensitized to radiation by vinorelbine using different schedules combining exposure to both. The sensitizer enhancement ratios (SERs) at a cell survival level of 10% were 1.42 ± 0.21 to 1.33 ± 0.06, and 1.22 ± 0.07 depending on schedule. Vinorelbine-induced radiosensitization did not depend on the schedule of the combined exposure. Flow cytometric analyses showed that the cells did not accumulate in the radiosensitive G_2/M phase of the cell cycle after concurrent treatment with vinorelbine and radiation. The results of an alkaline filter elution assay demonstrated that in the presence of vinorelbine at 1 n*M* radiation-induced DNA strand breaks were not completely repaired at 24 h postradiation. We conclude that human SCLC SBC-3 cells are sensitized to radiation by vinorelbine and that a possible mechanisms of vinorelbine-induced radiosensitization may at least in part be associated with impairment of DNA repair following radiation-induced DNA damage.

Keywords Vinorelbine · Radiosensitization · Small cell lung cancer cells · DNA repair

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Introduction

Vinorelbine (Navelbine, KW-2307), a semisynthetic vinca alkaloid, is a potent inhibitor of mitotic microtubule polymerization. Like other vinca alkaloids, vinorelbine is believed to exert its antitumor effects by binding to tubulin, the basic protein subunit of microtubules. This process inhibits microtubule assembly, resulting in the disruption of the mitotic spindle network and, ultimately, in cell cycle arrest in metaphase of tumor cell division [6]. Preclinical studies have shown that vinorelbine is active against small cell lung cancer (SCLC) as well as non-small cell lung cancer (NSCLC) [2, 7], and several clinical trials have been designed to evaluate the activity of vinorelbine for the treatment of SCLC [10].

Recent studies have provided evidence that vinorelbine can potentiate the antitumor effects of radiation, and act as a radiosensitizer against human NSCLC cells [3]. We have previously demonstrated that both prolonged G_2/M accumulation concomitant with continuous polyploidization and an increase in susceptibility to induction of apoptosis may be associated with the cellular mechanisms of radiosensitization produced by vinorelbine in human NSCLC cells [4]. However, little is known about vinorelbine-induced radiosensitization of human SCLC cells. The aims of this study were to demonstrate vinorelbine-induced radiosensitization of human SCLC SBC-3 cells and to elucidate the mechanisms of radiosensitization.

Materials and methods

Drug

Vinorelbine (Navelbine, VNR, KW-2307) was obtained from Kyowa Hakko Kogyo (Tokyo, Japan).

Cell lines and culture

The SCLC cell line SBC-3 was kindly donated by Dr. Kimura (Okayama University, School of Medicine, Okayama, Japan) [16].

The cells were maintained in RPMI-1640 at 37°C (Nikken Biomedical Laboratories, Kyoto, Japan) containing 10% heat-inactivated fetal bovine serum (FBS) (IBL, Fujioka, Japan), penicillin (100 U/ml), and streptomycin (100 μg/ml) in a humidified atmosphere containing 5% CO_2.

Drug and radiation exposure

As shown in Fig. 1, SBC-3 cells were exposed to the drug and radiation using three different schedules. In the first, SBC-3 cells were simultaneously exposed to vinorelbine at 1 n*M* and radiation and cultured in medium containing vinorelbine (schedule A, concurrent and continuous exposure). Other cells were treated with vinorelbine at 1 n*M* for 24 h prior to radiation, and after irradiation were cultured in medium containing vinorelbine at 1 n*M* (schedule B, VNR pretreatment and continuous exposure). Still other cells were treated with vinorelbine at 1 n*M* for 24 h prior to irradiation. After removing vinorelbine from the medium, the cells were irradiated, and then cultured in drug-free medium (schedule C, VNR pretreatment). Cells (1×10^4 per centrifuge tube) were irradiated at room temperature with a ^{60}Co γ-irradiator (Shimazu Company, Kyoto, Japan) at a dose rate of 0.64 Gy/min. Radiation doses were varied by changing the exposure time, and ranged from 1 to 6 Gy.

Clonogenic assay

Radiosensitivity was determined using the double-layer soft-agar clonogenic assay, as previously described [13]. Cells plated in the top layer which consisted of RPMI-1640 containing 10% FBS and 0.3% agar. A 1-ml volume per well was layered onto a previously prepared underlayer consisting of 0.5% agar in McCoy's medium 5A (Gibco BRL) enriched with 50 ml heat-inactivated horse serum, 4 ml of 2.2% sodium pyruvate, 4 ml 200 m*M* glutamine, 0.8 ml 2.1% serine, 4 ml 100 U/ml penicillin, and 100 μg/ml streptomycin per 400 ml medium. All assays were performed in triplicate using six-well plate dishes (Linbro, Va.), and the values obtained in one experiment are the means of triplicate assays. Plates were incubated at 37°C in a humidified atmosphere containing 5% CO_2. After 10 to 14 days, the colonies were counted with a CP-2000 automatic colony counter (Shiraimatsu Instrument, Osaka, Japan). Colonies larger than 50 μm in diameter were defined as "positive". The sensitizer enhancement ratios (SERs) were calculated by dividing the dose of radiation required to kill 90% of the control cells by the dose of radiation required to kill 90% of the cells exposed to vinorelbine [11]. The data were fitted by linear regression to a plot of log survival fraction versus radiation dose.

Flow cytometry

Cells (2×10^5/dish) were harvested by trypsinization, washed with ice-cold phosphate-buffered saline (PBS), fixed with ice-cold 70% ethanol solution, and stored at 4°C for at least 6 h. After removing the ethanol solution, the cell pellets were resuspended in 0.2 *M* Na_2HPO_4 and 0.1 *M* citrate (pH 7.4), hydrolyzed with 250 μg/ml ribonuclease (RNase) (Nippon Gene, Tokyo, Japan) at 37°C for 30 min, and stained with 50 μg/ml ethidium bromide. The DNA content of the cells was determined by flow cytometry using a FACScan flow cytometer (Becton Dickinson, San Jose, Calif.). The cell cycle distribution was calculated using CellQuest software and the ModFit program (Becton Dickinson).

Alkaline filter elution assay

Prior to filter elution assay, exponentially growing cells were radiolabeled by incubation with 0.1 μCi/ml of [methyl-^{14}C]thymidine (2.15 GBq/mmol; Amersham, Little Chalfont, UK) for 24 h. The cells were then washed with free of radioactive medium and incubated for at least 2 h before exposure to the drug and/or radiation. To determine the radiation-induced DNA single-strand breaks in SBC-3 cells, the alkaline filter elution procedure was performed as described previously [8]. Cells (1×10^5) were exposed to the drug and/or radiation in different schedules: vinorelbine alone at 1 n*M* for 24 h; radiation alone at a dose of 6 Gy and immediately subjected to the assay; radiation alone at a dose of 6 Gy and subjected to the assay 24 h later; or vinorelbine at 1 n*M* and radiation at a dose of 6 Gy simultaneously and subjected to the assay 24 h after irradiation.

After exposure to the drug and/or radiation, cells were placed on ice to arrest DNA repair, then diluted with ice-cold PBS and gently deposited onto a Nuclepore polycarbonate filter (pore size 2 μm; Costar, Cambridge, Mass.). The cells were lysed on the filter for 1 h with 5 ml lysis solution containing 2% sodium dodecyl sulfate, 25 m*M* EDTA, 50 m*M* Tris, 50 m*M* glycine, and 0.5 mg/ml proteinase K, pH 9.6. The lysis solution was allowed to flow through the filter under gravity, and the filter was then rinsed three times with 3 ml 20 m*M* disodium EDTA, pH 9.6, to remove most of the cell protein, membrane, and RNA. The remaining DNA (more than 97% of the amount applied to the filter) was analyzed by alkaline elution with tetrapropylammonium-tetrahydroxy-EDTA, pH 12.1, at a flow rate of 0.025 to 0.03 ml/min. The fractions of the eluted solution were directly collected into scintillation vials on a fraction collector at 1.5-h intervals for 15 h. The fractions were then mixed with five volumes of Clear-sol I (Nacalai Tesque, Kyoto, Japan) containing 0.5% (v/v) acetic acid, and the radioactivity was counted in an LS6000 liquid scintillation counter (Beckmann, Fullerton, Calif.).

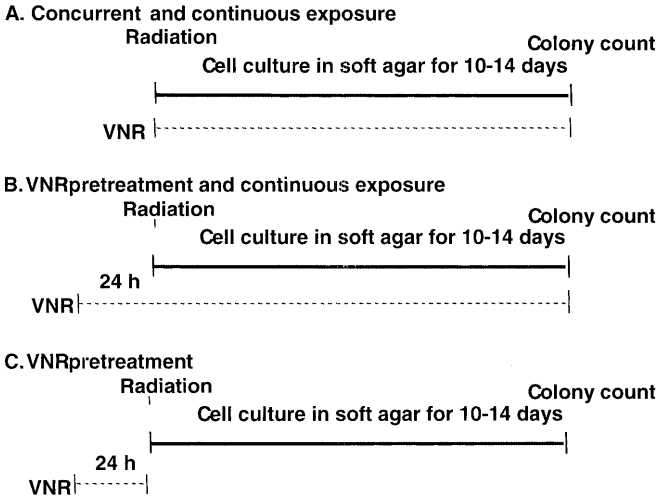

Fig. 1. Combination schedules of vinorelbine (*VNR*) and radiation. *Schedule A* Concurrent and continuous exposure: cells simultaneously exposed to VNR at 1 n*M* and radiation and then cultured in medium containing VNR. *Schedule B* VNR pretreatment and continuous exposure: cells exposed to VNR at 1 n*M* for 24 h prior to radiation, then irradiated and cultured in medium containing VNR at 1 n*M*. *Schedule C* VNR pretreatment: cells exposed to VNR at 1 n*M* for 24 h prior to radiation. After removal of VNR from the medium, the cells were irradiated and then cultured in drug-free medium

Results

Vinorelbine sensitizes human SCLC SBC-3 cells to radiation

To determine whether vinorelbine sensitized human SCLC SBC-3 cells to radiation, the cells were exposed to vinorelbine and radiation using the three different

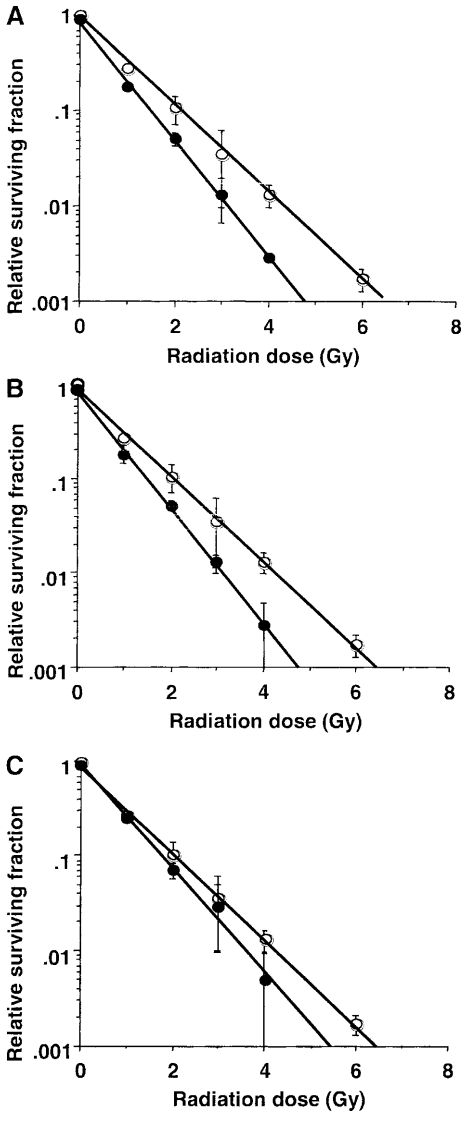

Fig. 2A–C. Effect of vinorelbine on the radiosensitivity of SBC-3 cells. Exponentially growing SBC-3 cells were not exposed to vinorelbine (*open circles*) or were exposed to 1 n*M* vinorelbine (*solid circles*). Radiation doses ranged from 1 to 6 Gy. A clonogenic assay was performed as described in Materials and methods. Clonogenic survival curves were constructed according to each schedules combining exposure to vinorelbine and radiation: **A** concurrent and continuous exposure, **B** vinorelbine pretreatment and continuous exposure, and **C** vinorelbine pretreatment. The sensitizer enhancement ratio (SER) was calculated by dividing the dose of radiation required to kill 90% of the control cells by the dose of radiation required to kill 90% of the cells exposed to vinorelbine. The data were fitted by linear regression to a plot of log survival fraction versus radiation dose. Values shown are the means ± SD (*bars*) from three independent experiments

schedules. Figure 2 shows the clonogenic survival curves of the SBC-3 cells irradiated at the doses indicated, with or without vinorelbine. The relative surviving fractions of SBC-3 cells treated with vinorelbine alone at 1 n*M* were more than 90% in all three schedules and thus at 1 n*M* vinorelbine had hardly any effect on the clonogenic survival of SBC-3 cells. As shown in Fig. 2, SBC-3 cells were sensitized to radiation by vinorelbine at 1 n*M* by three combined-exposure schedules. The SERs at 10% cell survival were 1.42 ± 0.21 with schedule B, 1.33 ± 0.06 with schedule A, and 1.22 ± 0.07 with schedule C. Vinorelbine-induced radiosensitization did not depend on the combined-exposure schedule, because there were no significant differences in the SERs at the 10% cell survival level between schedule A, B, and C. We therefore investigated the mechanisms of vinorelbine-induced radiosensitization in the cells exposed to vinorelbine and radiation simultaneously.

Effects of vinorelbine and radiation on cell cycle distribution of human SCLC SBC-3 cells

We have previously demonstrated that the cellular mechanisms of vinorelbine-induced radiosensitization in human NSCLC cells may involve both prolonged G_2/M accumulation concomitant with continuous polyploidization and an increase in susceptibility to induction of apoptosis. To determine whether these mechanisms are involved in vinorelbine-induced radiosensitization of SBC-3 cells, we examined the effects of vinorelbine and/or radiation on the cell cycle distribution of SBC-3 cells by flow cytometry (Fig. 3). Untreated control cells continued to divide throughout the 24-h time course of the experiment and the percentages of cells in the G_0/G_1 phase (33%), S phase (42%), and G_2/M phase (25%) were essentially unchanged from 6 h to 24 h. The cell cycle distribution of untreated control cells was compared with that of cells exposed to vinorelbine alone at 1 n*M*, cells exposed to radiation alone at a dose of 6 Gy, and cells simultaneously exposed to vinorelbine at 1 n*M* and radiation at a dose of 6 Gy. The cell cycle distribution of the cells exposed to vinorelbine alone at 1 n*M* did not change from 6 h to 24 h. In the cells exposed to radiation alone at a dose of 6 Gy, S phase delay and G_2 phase blockage of the cell cycle were observed at 6 h and 24 h after irradiation, respectively. There appeared to be no differences in the cell cycle distribution between the cells exposed to radiation alone and the cells simultaneously exposed to vinorelbine and radiation. Thus, accumulation of cells in the radiosensitive G_2/M phase of the cell cycle did not account for the vinorelbine-induced radiosensitization of SBC-3 cells.

DNA strand breaks induced by vinorelbine and/or radiation in human SCLC SBC-3 cells

We speculate that another possible mechanism for vinorelbine-induced radiosensitization may be impairment of DNA repair following radiation-induced DNA damage. Since we had previously reported that radiation-induced DNA strand breaks can be identified by the alkaline filter elution assay [8], we used it in this study to determine whether vinorelbine inhibited repair of radiation-induced DNA damage in SBC-3 cells (Fig. 4). When DNA strand breaks have been induced, DNA can pass through the filter, resulting in a decrease in the

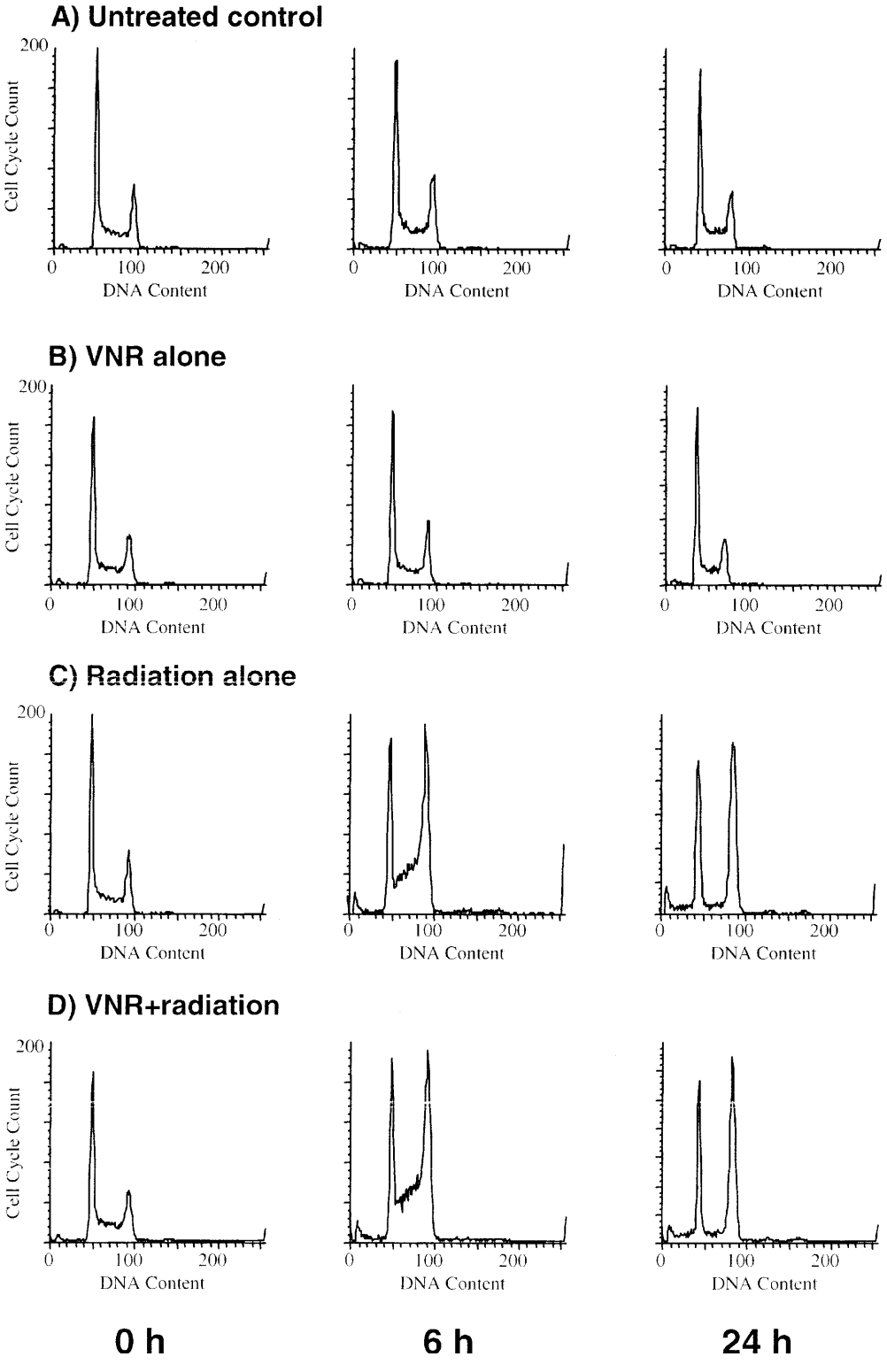

Fig. 3A–D. Effect of vinorelbine and/or radiation on the cell cycle distribution in SBC-3 cells. DNA staining and a flow cytometric analysis of SBC-3 cells were performed as described in Materials and methods. The cell cycle distributions from 6 h to 24 h after treatment with vinorelbine and/or radiation are shown. **A** Untreated control cells; **B** cells exposed to vinorelbine alone at 1 n*M*; **C** cells exposed to radiation alone at a dose of 6 Gy; **D** cells simultaneously exposed to vinorelbine at 1 n*M* and radiation at a dose of 6 Gy

amount of DNA retained on it. As shown in Fig. 4, induction of DNA strand breaks was demonstrated when the cells were irradiated at a dose of 6 Gy and immediately subjected to the assay. However, the radiation-induced DNA strand breaks appeared to have been almost completely repaired by 24 h after irradiation because there was no significant difference in the amount of DNA retained on the filter between the irradiated cells and untreated control cells. Treatment with vinorelbine alone at 1 n*M* for 24 h apparently did not induce DNA strand breaks. The finding that in the presence of vinorelbine at 1 n*M*, the radiation-induced DNA strand breaks were not completely repaired by 24 h after irradiation was particularly interesting

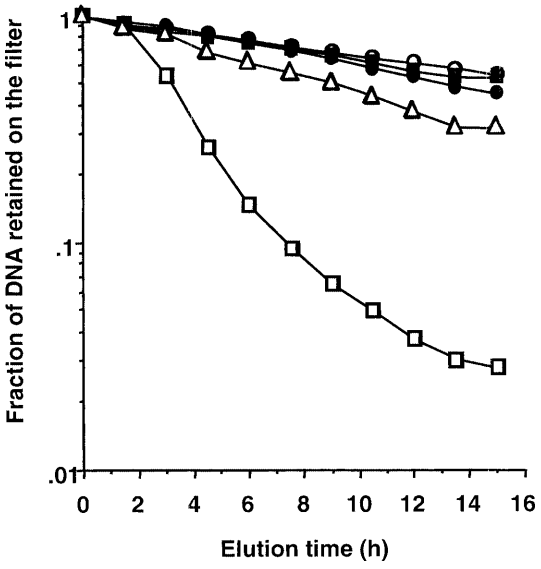

Fig. 4. DNA strand breaks induced by vinorelbine and/or radiation in human SCLC SBC-3 cells. DNA strand breaks were determined by the alkaline filter elution assay performed as described in Materials and methods. The fraction of ^{14}C-labeled DNA retained on the filter was plotted against the elution time (*open circles* untreated control cells, *solid circles* cells exposed to vinorelbine alone at 1 n*M* for 24 h; *open squares* cells exposed to radiation alone at a dose of 6 Gy and immediately subjected to the assay; *solid squares* cells exposed to radiation alone at a dose of 6 Gy and subjected to the assay 24 h later; *open triangles* cells simultaneously exposed to vinorelbine at 1 n*M* and radiation at a dose of 6 Gy and subjected to the assay 24 h after irradiation)

because it indicated that inhibition of repair of radiation-induced DNA strand breaks in SBC-3 cells may be at least in part associated with the mechanisms of vinorelbine-induced radiosensitization.

Discussion

In the present study we clearly demonstrated that a low cytotoxic dose of vinorelbine can sensitize human SCLC SBC-3 cells to radiation. Vinorelbine-induced radiosensitization did not depend on the combined-exposure schedule. It is well accepted that an accumulation of cells in the radiosensitive G_2/M phase of the cell cycle accounts for the radiosensitization produced by antimicrotubule agents such as paclitaxel and vinorelbine [1, 3, 4, 11]. By contrast, several studies have shown that paclitaxel-induced radiosensitization is not cell-cycle-dependent [15] and that tumor reoxygenation is the mechanism of paclitaxel-induced enhancement of the in vivo tumor radioresponse [12]. Flow cytometric analysis showed that the combination of vinorelbine and radiation did not induce accumulation of cells in the G_2/M phase, which is believed to be the most radiosensitive cell cycle phase.

These findings and the present findings prompted us to speculate that another mechanism in addition to mitotic arrest was responsible for the vinorelbine-induced radiosensitization of human SCLC cells. The alkaline filter elution assay showed that radiation-induced DNA strand breaks were not completely repaired in the presence of vinorelbine. This observation indicated that inhibition of repair of radiation-induced DNA strand breaks in SBC-3 cells may be at least in part associated with the mechanisms of vinorelbine-induced radiosensitization. However, we failed to demonstrate that vinorelbine directly inhibited the repair of radiation-induced DNA strand breaks in SBC-3 cells.

It has been well documented that p53 plays an important role in the DNA repair machinery, including nucleotide excision repair [9]. The C-terminal domain of p53 is thought to be involved in the recognition of damaged DNA [14, 18]. We have previously demonstrated the nuclear accumulation of p53 and the induction of p21 in response to radiation-induced DNA damage in SBC-3 cells expressing functional p53 [17]. Recent studies have provided evidence that functional microtubules and the dynein motor protein participate in the transport of p53, and facilitate its nuclear accumulation to repair damaged DNA [5]. Treatment with antimicrotubule agents before DNA damage reduces nuclear accumulation of p53 and expression of mdm2 and p21 [5]. Considered together, this line of evidence led us to hypothesize that the disruption of microtubule integrity in SBC-3 cells by vinorelbine may in part inhibit p53 transport to nucleus, resulting in impairment of p53-mediated DNA repair following radiation-induced DNA damage. Further studies are required to elucidate the molecular mechanisms by which vinorelbine induces radiosensitization through the impairment of DNA repair.

In conclusion, our preliminary results suggest that vinorelbine can sensitize human SCLC SBC-3 cells to radiation at least in part by impairing DNA repair following radiation-induced DNA damage. We expect the combination of vinorelbine and radiotherapy to be a promising treatment for patients with limited-stage SCLC.

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