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ORIGINAL ARTICLE

Pretreatment with 5-FU enhances cisplatin cytotoxicity in head and neck squamous cell carcinoma cells

Kei Ijichi · Makoto Adachi · Yasuhisa Hasegawa · Tetsuya Ogawa · Hideaki Nakamura · Ayumi Kudoh · Yoshihiro Yasui · Shingo Murakami · Kanji Ishizaki

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Abstract
Purpose In the treatment of head and neck malignancy, cisplatin and 5-FU have been used the most as chemotherapeutic agents. The difference in efficacies of these is unclear and controversial. To investigate more effective schedule, we analyzed the cytotoxicity in different treatment sequence with two agents in vitro and the mechanism for different effectiveness.
Methods UM-SCC-23 and UM-SCC-81B, head and neck squamous cell carcinoma cell lines, were analyzed for cellular killing in alternative sequence treatment with cisplatin and 5-FU. The treatment schedule was designed based on the clinical regimen. To determine the mechanism for the difference of cytotoxicity with each schedule, cell cycle distributions of both cells after 5-FU treatment with various durations were analyzed by flow-cytometry and immunostaining with anti-PCNA and anti-BrdU.
Results 5-FU pretreatment followed by cisplatin treatment showed higher cell killing in both types of cells than the reverse treatment schedule. In the cell cycle analysis and immunostaining after the treatment of 5-FU, the rate of PCNA-positive cells was increased from 24 to 144 h in both cells. The rate of BrdU-positive cells of UM-SCC-81B in flow-cytometry was also increased, while that of UM-SCC-23 was gradually decreased. These data suggested that the cells treated with 5-FU for more than 144 h were still in the S-phase with or without DNA synthesis.
Conclusions In head and neck carcinoma cells, we showed 5-FU pretreatment enhanced cisplatin cytotoxicity. The result of cell cycle analysis and immunostaining showed S-phase arrest by treatment of prolonged 5-FU treatment. The very long arrest in S-phase might be a mechanism to enhance cisplatin cytotoxicity by 5-FU pretreatment. We thus suggest pretreatment with 5-FU to enhance the effectiveness of cisplatin-based chemotherapy.

Keywords HNSCC · Chemotherapy · Cisplatin · 5-FU · Cell cycle

K. Ijichi (✉) · M. Adachi · H. Nakamura · Y. Yasui · K. Ishizaki
Central Laboratory and Radiation Biology,
Aichi Cancer Center Research Institute,
1-1 Kanokoden, Chikusa-ku, Nagoya, Japan
e-mail: ijichi@aichi-cc.jp

K. Ijichi · S. Murakami
Department of Neuro-Otolaryngology,
Nagoya City University Graduate School of Medical Sciences,
1 Kawasumi, Mizuho-cho, Mizuho-ku, Nagoya, Japan

Y. Hasegawa · T. Ogawa
Department of Head and Neck Surgery,
Aichi Cancer Center Hospital,
1-1 Kanokoden, Chikusa-ku, Nagoya, Japan

A. Kudoh
Division of Virology, Aichi Cancer Center Research Institute,
1-1 Kanokoden, Chikusa-ku, Nagoya, Japan

Introduction

Cisplatin and 5-fluorouracil (5-FU) have been widely used in the treatment of human cancer. In combination with various other chemotherapeutic agents, 5-FU also improves response rates and survival in breast, colorectal and head and neck cancers [1]. In head and neck cancer chemotherapy, mainly regimens with a combination of 5-FU and platinum agent have been used [2]. Recently, the efficacy of a new chemotherapeutic regimen, which adds docetaxel to the combination of cisplatin and 5-FU, is under clinical study [3].

5-FU is an analogue of uracil and rapidly enters in to cells using the same facilitated transport mechanism as uracil. Then, it is converted intracellularly to several active metabolites. A major mechanism of 5-FU cytotoxicity is thought to be the inhibition of thymidylate synthase (TS) by FdUMP, although the exact molecular mechanisms of TS inhibition are still unclear. Inhibition of TS causes depletion of dTMP resulting in subsequent depletion of dTTP, which induces perturbations in the levels of the other deoxynucleotide levels (dATP, dGTP and dCTP) through various feedback mechanisms. Deoxynucleotide pool imbalances (in particular, the dATP/dTTP ratio) might also inhibit DNA synthesis and repair [1, 4].

The cellular target of cisplatin is generally acknowledged to be DNA. Once cisplatin is transported into cells, it binds to DNA, inducing various types of inter- and intrastrand cross-links. Cisplatin-damaged DNA causes cell-cycle perturbation such as arrest in the G2-phase and in the absence of adequate repair, the cells eventually undergo an abortive attempt at mitosis that results in cell death via an apoptotic mechanism [5].

In previous studies, the cytotoxicity and efficacy of the chemotherapeutic regimen with a combination of cisplatin and 5-FU were reported using experimental and clinical approaches. Although, a few analyses of the treatment sequence of these two agents were reported, they were limited to shorter durations of 5-FU and cisplatin treatment, or to treatment only with cisplatin-resistant cells [6–10].

To find a more effective treatment sequence of the two agents, we designed an alternative model of 5-FU and cisplatin for the treatment of head and neck squamous cell carcinoma (HNSCC) cells in vitro, based on a clinical treatment regimen for HNSCC. As a result, we found higher cytotoxicity in a schedule in which 5-FU was first given, followed by cisplatin, rather than the reverse. We also performed cell cycle analysis after exposure to 5-FU for various durations and showed S-phase accumulation of these cells. These results suggested that 5-FU pretreatment enhanced the cytotoxicity of the subsequent cisplatin treatment.

Materials and methods

Cells and cell culture

The UM-SCC-23 and UM-SCC-81B cells (HNSCC cell lines) were kindly donated by Dr. Thomas E. Carey, Laboratory of Head and Neck Cancer Biology at the University of Michigan. This cell line was maintained in Dulbecco's modified Eagle's medium (DMEM; Sigma, MO, USA) supplemented with 10% fetal bovine serum (FBS; Invitrogen, CA, USA) in a humidified atmosphere of 5% CO_2 at 37°C.

Colony formation assay

The appropriate amount of cells were inoculated in a 6 cm dish, treated with each concentration of cisplatin for 24 h or 5-FU for 6 days, then incubated for approximately 7–14 days and stained with crystal violet. Colonies containing more than 50 cells were scored. On the basis of these results, we then determined the dose of each drug in the following experiments. UM-SCC-23 and UM-SCC-81B cells were treated according to the following two schedules (Fig. 1). Schedule A: 5.0×10^4–1.0×10^6 cells were inoculated in 10 cm dishes and next day 5-FU was added and cultured. On day 3, the culture medium was changed to a fresh one with 5-FU. After 6 days of culture, the cells were washed twice with PBS and then 2.0×10^2–1.0×10^5 cells were placed in 6 cm dishes. After incubation overnight, cells were incubated with cisplatin for 24 h. Schedule B: 1.0×10^2–1.0×10^5 cells were inoculated into 6 cm dishes and incubated overnight, cisplatin was added for 24 h, and then cells were washed twice with PBS and cultured again with 5-FU for another 6 days. The culture medium with 5-FU was refreshed on day 4. When further culture was required, medium was changed to a fresh one without agents, after washing twice with PBS and incubation was continued. The colony was stained and scored as described above. In addition, to distinguish the two schedules, control schedule was the same but without cisplatin.

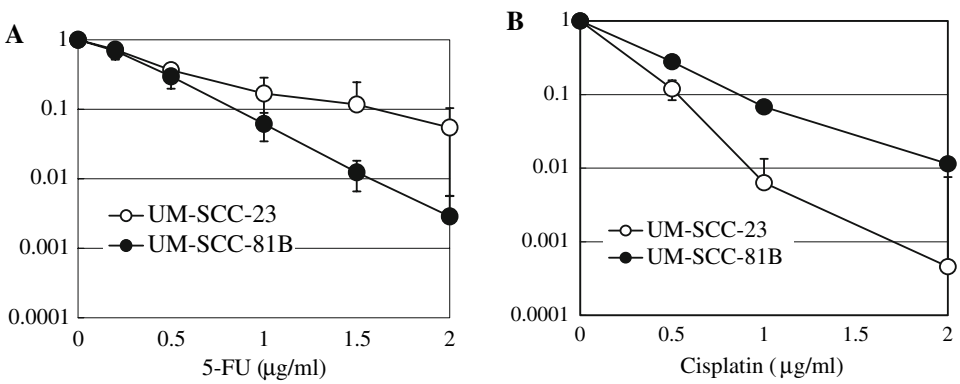

Fig. 1 Sensitivities of HNSCC cells UM-SCC-23 and UM-SCC-81B to 5-FU or cisplatin. Both cell lines were treated with increasing concentrations of **a** 5-FU for 144 h and **b** cisplatin for 24 h. Cytotoxicity analysis was performed by colony formation assay. Each data point is the mean of three independent experiments. *Vertical bars* standard deviations

Cell cycle analysis using flow cytometry

Cell cycle distribution of both cells after 5-FU or cisplatin treatment was determined by two-parameter flow cytometrical analysis: PI (DNA content) and BrdU uptake [11]. After 5-FU or cisplatin treatment for various durations, cells were labeled with 20 μM BrdU for 30 min and harvested. Then, following the manufacturer's protocol, nuclei were isolated and treated with 2 N HCl. Nuclei in 50 μl of PBS-TB (10% PBS containing Tween 20 and BSA) were treated with anti-BrdU antibody (Becton Dickinson, San Jose, CA, USA) followed by Alexa fluor 488 goat anti-mouse antibody (Molecular Probes, Eugene, OR, USA). Nuclei were counterstained with 50 μg/ml PI, filtered through Cell-Strainer (Becton Dickinson) and then analyzed with a FACScan cytometer (Becton Dickinson) using Cell Quest software.

Detection of PCNA and BrdU incorporation by immunofluorescence microscopy

Approximately 1.0×10^5 cells were grown on coverslips in 6 cm dishes. Cells were incubated with 5-FU for 24–144 h. At the indicated time, BrdU was added in culture medium to a final concentration of 20 μM and incubated for 30 min. After treatment, the slides were fixed with 2% paraformaldehyde and washed twice with PBS. Slides were incubated with 100% methanol at −20°C for 5 min, and washed twice with PBS. For anti-BrdU antibody staining, the slides were incubated with 4 N HCl at room temperature for 30 min. After washing twice with PBS, the slides were incubated with anti-BrdU or anti-PCNA antibody (Becton Dickinson) and then secondary antibody. More than 1,000 cells were scored for positive and negative nuclei. For each incubation time, percentages of positive cells were determined.

Statistical analysis

Statistical analysis was performed using the JMP software (version 6.0, SAS Institute Inc., NC, USA). The differences were considered statistically significant when the $p < 0.05$.

Results

Cytotoxicity of cisplatin or 5-FU

The respective sensitivities of the UM-SCC-23 and UM-SCC-81B cells to cisplatin and 5-FU toxicity were determined by colony formation assay (Fig. 1). With 24 h cisplatin treatment, UM-SCC-23 cells were approximately twofold more sensitive than UM-SCC-81B cells. In contrast, UM-SCC-81B cells were approximately twofold more sensitive than UM-SCC-23 cells to the 6 days treatment with 5-FU (Fig. 2).

Cytotoxicity of cisplatin and 5-FU with alternate treatment schedule

The respective cellular sensitivities of UM-SCC-23 cells and UM-SCC-81B cells to combination treatment with cisplatin and 5-FU according to the alternative schedule are shown in Fig. 3. Treatment doses were determined by the sensitivity of both cells to cisplatin and 5-FU (Fig. 1). We used doses of cisplatin, which give a survival rate of 0.1, namely 1.0 μg/ml for UM-SCC-23 cells and 2.0 μg/ml for UM-SCC-81B cells. A dose of 5-FU ranged from 0.2 to 1.0 μg/ml for UM-SCC-23 cells, and from 0.1 to 0.4 μg/ml for UM-SCC-81B cells. The treatment schedule with combined cisplatin and 5-FU was designed with reference to the protocol for clinical treatment of head and neck cancer. Schedule A with 5-FU treatment followed by cisplatin, showed significant higher cell killing of both cells than schedule B with the reverse treatment (UM-SCC-23, $p = 0.0013$; UM-SCC-81B, $p < 0.0001$), and the difference in cytotoxicity between these two schedules increased in proportion to the 5-FU concentration. The cytotoxic effects of control experiments with 5-FU alone, showed no difference in both schedules for both cells.

Cell cycle distribution after treatment by 5-FU or cisplatin

To ascertain the mechanism of sensitization to cisplatin by 5-FU pretreatment, we analyzed the cell cycle distribution after 5-FU or cisplatin treatment of both cells. The concentration of 5-FU was fixed at 1.0 μg/ml for UM-SCC-23 cells and 0.4 μg/ml for UM-SCC-81B cells. For cisplatin, it was 1.0 μg/ml for UM-SCC-23 cells and

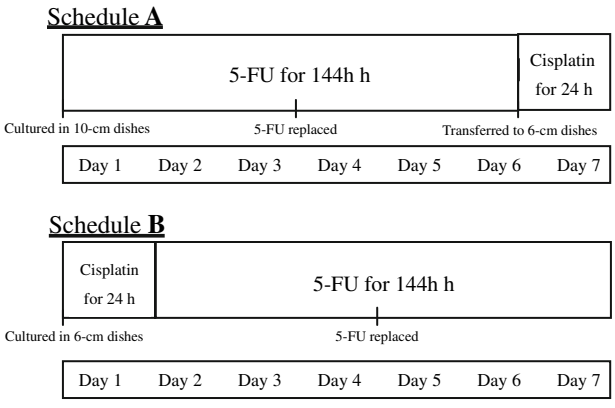

Fig. 2 Schedules for 5-FU and cisplatin treatment in this study. Alternative 5-FU and cisplatin treatment schedule models (Schedule A and B) were modifications of clinical treatment schedules

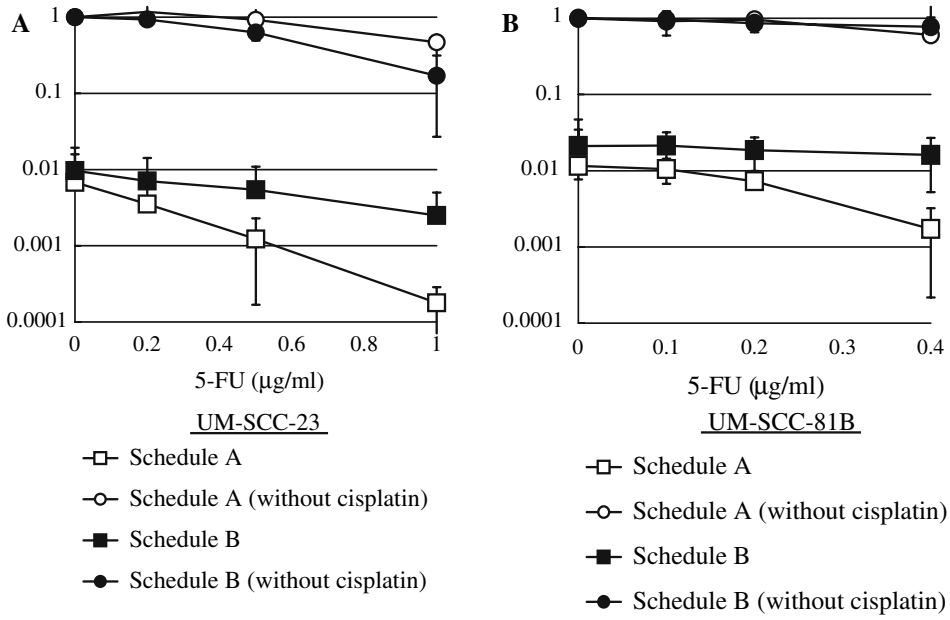

Fig. 3 Sensitivities of HNSCC cells to alternative schedules of 5-FU and cisplatin treatment. **a** UM-SCC-23 cells and **b** UM-SCC-81B cells were treated with increasing concentrations of 5-FU for 144 h and a fixed concentration of cisplatin for 24 h. Cytotoxicity analysis was performed by colony formation assay. Cisplatin concentrations were determined as to give 10% survival rate by the single agent treatment. Each data point is the mean of three independent experiments. *Vertical bars* standard deviations

2.0 μg/ml for UM-SCC-81B cells. These doses exhibited the largest difference in cytotoxicity between A and B treatment schedules. At the indicated time, BrdU was incorporated and analyzed by flow cytometry (Fig. 4). After treatment with 5-FU, S-phase cells of UM-SCC-23 were increased and the rate of S-phase peak was more than 80% at 24 h and then it gradually decreased till 144 h UM-SCC-23. In the dot-plot profiles of flow cytometry analysis of UM-SCC-23 cells, 2–10% of cells were distributed between 2N and 4N of DNA content at 72 and

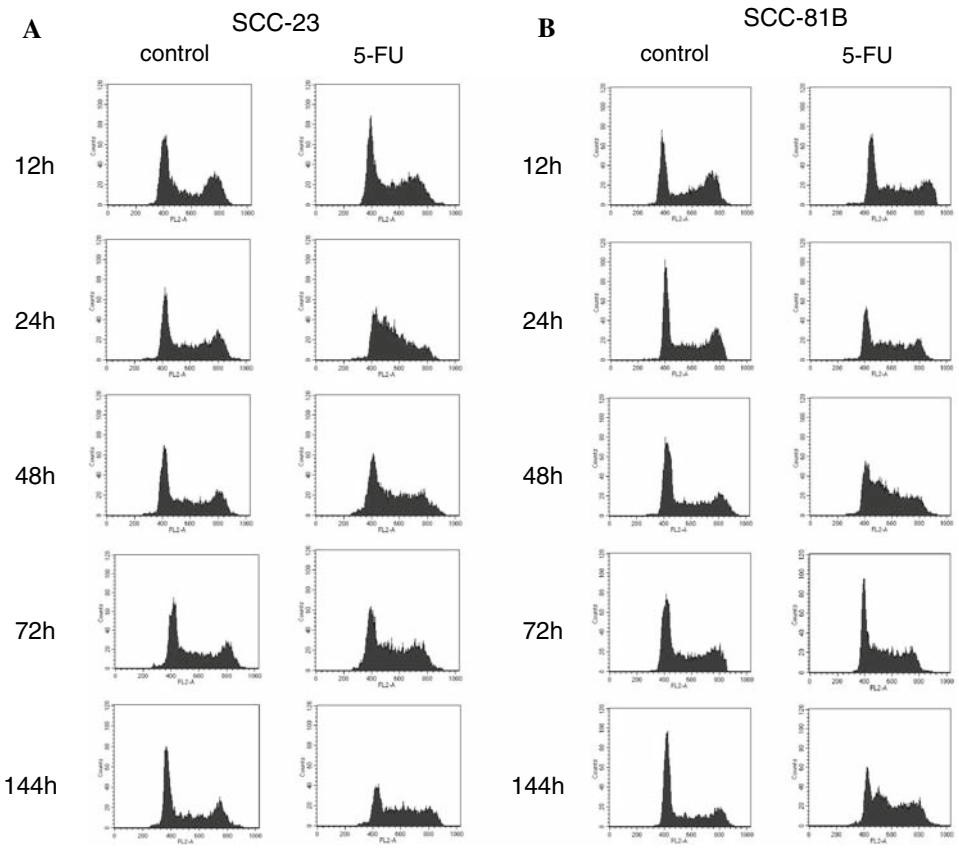

Fig. 4 Cell cycle distribution of HNSCC cells after 5-FU treatment for various durations. The **a** UM-SCC-23 cells and **b** UM-SCC-81B cells were analyzed using a flow cytometer after 12–144 h 5-FU treatment

Table 1 S-phase distribution in HNSCC cells after 5-FU treatment for various durations

Duration of treatment (h)	S-phase accumulation (%)								
	5-FU					Control			
	Early	Middle	Late	Total	Others*	Early	Middle	Late	Total
UM-SCC-23									
12	29.63 ± 3.53	20.81 ± 1.07	18.56 ± 3.85	67.82 ± 0.99	–	29.44 ± 5.38	17.29 ± 7.14	13.19 ± 0.09	58.80 ± 11.46
24	37.89 ± 11.22	35.01 ± 5.91	15.76 ± 5.67	87.05 ± 0.28	–	22.70 ± 5.12	21.24 ± 1.27	21.73 ± 2.53	64.19 ± 0.56
48	35.76 ± 0.39	22.85 ± 2.56	14.57 ± 0.71	73.17 ± 5.11	–	24.90 ± 1.97	18.62 ± 1.73	15.54 ± 0.93	58.58 ± 1.27
72	26.00 ± 0.53	23.39 ± 2.14	17.80 ± 0.62	66.75 ± 1.68	2.39 ± 0.35	23.21 ± 3.07	19.59 ± 2.58	14.34 ± 1.41	56.40 ± 0.82
144	15.82 ± 7.13	18.82 ± 0.72	9.89 ± 2.61	44.09 ± 9.78	9.26 ± 5.76	19.46 ± 1.13	13.86 ± 3.30	11.72 ± 1.46	44.31 ± 3.52
UM-SCC-81B									
12	23.95 ± 3.13	19.23 ± 1.56	17.43 ± 3.44	59.63 ± 0.59	–	18.74 ± 4.47	17.84 ± 0.48	16.53 ± 4.13	53.69 ± 0.48
24	22.35 ± 1.97	21.48 ± 3.93	20.29 ± 3.90	63.79 ± 2.81	–	18.41 ± 1.38	17.00 ± 0.66	17.32 ± 5.30	52.27 ± 5.84
48	31.48 ± 11.08	26.95 ± 1.00	15.21 ± 4.60	73.01 ± 3.61	–	19.03 ± 1.74	18.86 ± 4.72	16.24 ± 2.14	53.40 ± 4.91
72	34.54 ± 2.38	26.85 ± 5.30	14.78 ± 3.42	76.43 ± 7.98	–	17.98 ± 1.87	16.84 ± 0.28	16.09 ± 2.57	56.60 ± 4.41
144	26.51 ± 2.62	28.79 ± 5.49	17.48 ± 2.61	73.14 ± 1.03	–	19.50 ± 0.06	16.16 ± 4.02	12.60 ± 4.13	48.22 ± 0.86

The UM-SCC-23 cells (A) and UM-SCC-81B cells (B) were analyzed by two parameters BrdU uptake and DNA content, and BrdU-positive cells were divided into early-, middle-, and late-S-phases by DNA content

* Cells distributed between 2 and 4N of DNA content but BrdU-negative

144 h, respectively, but negative for BrdU staining (Table 1). Also, S-phase cells of UM-SCC-81B were increased gradually from 24 to 72 h and the highest rate of S-phase peak was approximately 80%. It decreased at 144 h to the same rate of 48 h (Table 1). This suggests that the cell cycle progression after prolonged treatment with 5-FU may be delayed at S-phase due to difficulty of DNA synthesis.

After cisplatin treatment for 24 h, UM-SCC-23 cells accumulated to G1- and early S-phase cells (Table 2), while UM-SCC-81B cells accumulated to early or middle S-phase (Table 2). Furthermore, after cisplatin treatment even for 48 h, cell-cycle distributions were similar to those after 24 h treatment in both cells. These results suggest that DNA damage of cisplatin induced G1- or early/middle S-phase. It is estimated that progression of DNA synthesis is inhibited while cells are repairing DNA damage.

Expression of PCNA protein and BrdU uptake after 5-FU treatment

To characterize the cells with DNA content between 2 and 4 N at 144 h after 5-FU treatment, we performed immunostaining with anti-PCNA-antibody and anti-BrdU-antibody after treatment with 1.0 μg/ml 5-FU for UM-SCC-23 cells and 0.4 μg/ml 5-FU for UM-SCC-81B cells, followed by BrdU incorporation. The ratio of positive cells at each time of 5-FU treatment was shown in Fig. 6. The frequencies of BrdU-positive cells were consistent with those in flow cytometry. However, the PCNA-positive cells still maintained high number after 72 h till 144 h in UM-SCC-23

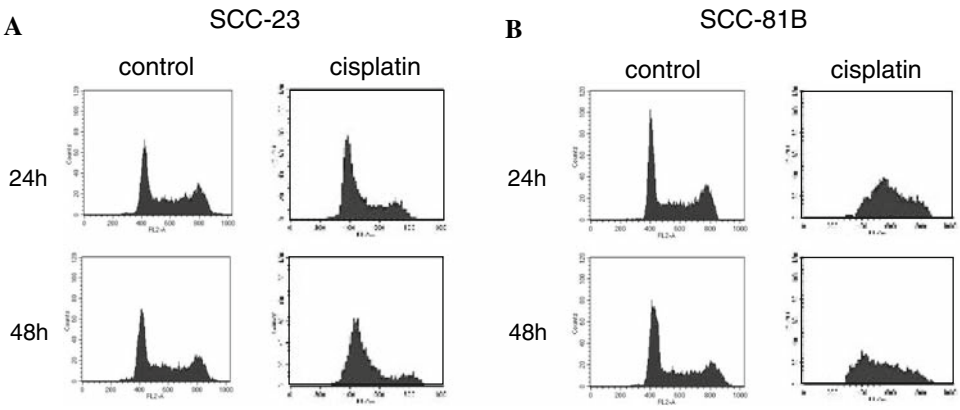

Fig. 5 Cell cycle distribution of HNSCC cells after cisplatin treatment for 24 and 48 h. **a** UM-SCC-23 cells and **b** UM-SCC-81B cells were analyzed using a flow cytometer after 24 or 48 h cisplatin treatment

Table 2 S-phase distribution of HNSCC cells after cisplatin treatment for 24 h or 48 h

Duration of treatment (h)	S-phase accumulation (%)							
	Cisplatin				Control			
	Early	Middle	Late	Total	Early	Middle	Late	Total
UM-SCC-23								
24	43.70 ± 2.33	15.73 ± 2.97	10.02 ± 0.81	69.42 ± 4.45	22.70 ± 5.12	21.24 ± 1.27	21.73 ± 2.53	64.19 ± 0.56
48	37.51 ± 0.92	10.99 ± 0.89	5.34 ± 0.70	52.79 ± 3.90	24.90 ± 1.97	18.62 ± 1.73	15.54 ± 0.93	58.58 ± 1.27
UM-SCC-81B								
24	42.62 ± 3.55	32.69 ± 2.06	9.65 ± 0.69	84.71 ± 0.68	18.41 ± 1.38	17.00 ± 0.66	17.32 ± 5.30	52.27 ± 5.84
48	47.60 ± 0.69	23.54 ± 0.38	11.84 ± 0.57	79.06 ± 0.26	19.03 ± 1.74	18.86 ± 4.72	16.24 ± 2.14	53.40 ± 4.91

The UM-SCC-23 cells (A) and UM-SCC-81B cells (B) were analyzed by two parameters BrdU uptake and DNA content, and BrdU-positive cells were divided into early-, middle-, and late-S-phases by DNA content

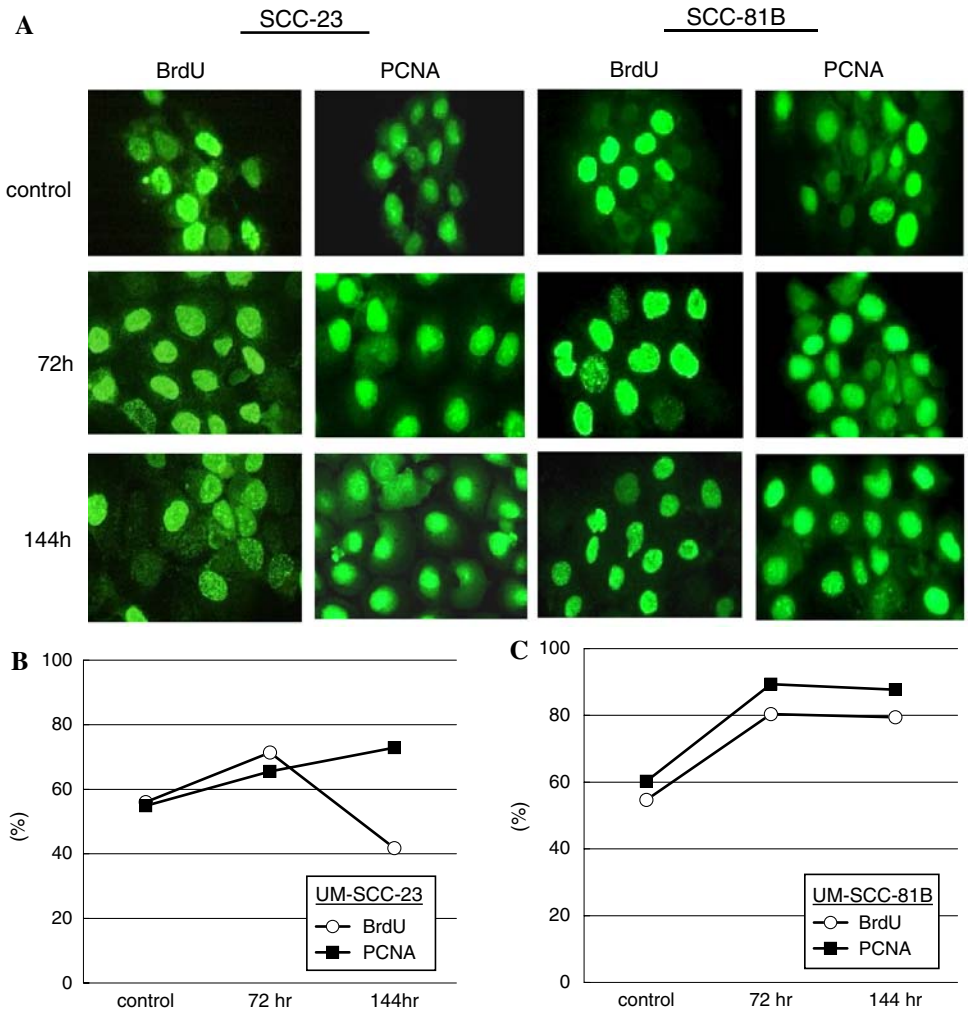

Fig. 6 Immunocytochemistry with anti-BrdU or anti-PCNA antibody of HNSCC cells after 5-FU treatment. **a** The UM-SCC-23 and UM-SCC-81B cells were immunostained with anti-BrdU or anti-PCNA antibody after 5-FU treatment. Durations of 5-FU treatment were 72 and 144 h, respectively. More than 1,000 cells were scored, and the positive rate was determined in **b** UM-SCC-23 and **c** UM-SCC-81B

cells. Also, in UM-SCC-81B cells, the frequency of both BrdU- and PCNA- positive cells after 72 and 144 h remained at high rate. This result showed that the frequency of the cells in S-phase increased depending on the duration of 5-FU treatment, although some of them might be in S-phase without DNA synthesis because of too long arrest.

Discussion

Although the chemotherapeutic regimen combining cisplatin and 5-FU has been widely used in head and neck cancer treatment, there have been many treatment variations of these agents in each institution in terms of the dose,

sequence and route of treatment [2]. For an effective treatment method, we have investigated the sequence in treatment with the two agents and analyzed their cytotoxicity using alternative treatment schedules.

In previous studies, the treatment sequence with 5-FU pretreatment before cisplatin showed an increased cytotoxicity to human cancer cells in vitro. However, these studies were limited to shorter duration of 5-FU and cisplatin treatment and involved only cisplatin-resistant cell lines. Another study analyzed the effective point of cisplatin administration during 5-FU treatment, but did not compare the effects of sequential treatment with the two agents [6, 7, 9, 10]. Furthermore, one of these studies with cisplatin-resistant cells concluded that since the intracellular glutathione level was reduced by 5-FU treatment, the repair of the platinum-DNA adduct by the following cisplatin treatment was inhibited [6]. Another report indicated that the cellular glutathione level was not decreased after 5-FU treatment [10]. Therefore, the mechanism of increased cisplatin sensitization by 5-FU pretreatment is still unclear. In the present study, we analyzed the cell cycle distribution after treatment of two squamous cell cartinoma cell lines with 5–FU. These results suggest that some cells in S-phase were without DNA synthesis after prolonged 5–FU treatment (Table 1). To confirm this hypothesis, we performed immunostaining with anti-PCNA antibody and anti-BrdU antibody. The BrdU and PCNA positive rates of 72 h treated cells were increased compared to control cells without treatment. After 144 h treatment, the BrdU-positive rate was decreased, but the PCNA positive rate was still increased in UM-SCC-23 cells (Fig. 6b). In UM-SCC-81B cells, both of BrdU- and PCNA-positive rates were increased after 72 and 144 h treatment (Fig. 6c). This result showed that the BrdU uptake of the cells, which were treated with 5-FU for 144 h in UM-SCC-23 cells, was decreased; yet, these cells were still in the DNA synthesis-phase. Besides, in UM-SCC-81B, the cells were accumulated in the DNA synthesis phase after 144 h treatment of 5-FU. Previous studies had reported that the cancer cells were accumulated in S-phase or made S-phase progression slowly after 5-FU treatment [12–15]. Thymidylate synthase (TS) inhibition reportedly depleted dTTP, thereby causing a dNTP imbalance [4, 16, 17], a major cytotoxic effect of 5-FU. After 5-FU treatment cells accumulate in S-phase and progresses slowly because of the dNTP imbalance. This mechanism has similarities with the results of the present study. In the dNTP imbalance following 5-FU treatment, DNA synthesis and also repair of platinum-DNA adduct by cisplatin might be severely affected. Thus, it was hypothesized that 5-FU treated cells which progress to the S-phase with marked dNTP imbalances would be less capable of repairing DNA damage [4]. Besides, the cell cycle analysis after cisplatin treatment, G1- or early and middle S-phase arrest resulted (Table 2). We considered that this G1 or early/middle S-phase arrest was caused by stopping the progress of DNA synthesis to repair cisplatin-induced DNA damage. When the dNTP imbalance was caused by 5-FU treatment after cisplatin pretreatment, the synergistic effect of 5-FU might be low at the status of inhibited DNA synthesis. Moreover, since at that time the DNA repair of cisplatin-induced DNA damage might have almost completed, the 5-FU-induced dNTP imbalance might be too late to inhibit the repair of it.

We concluded that this is one of the mechanisms by which prior 5-FU treatment followed by cisplatin demonstrates a higher cytotoxicity than the reverse sequential treatment. These results indicate that prior 5-FU treatment must well clinically enhance the subsequent cisplatin treatment, underscoring the more effective treatment sequence of 5-FU and cisplatin-based chemotherapy.

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References

1. Longley DB, Harkin DP, Johnston PG (2003) 5-fluorouracil: mechanisms of action and clinical strategies. Nat Rev Cancer 3(5):330–338
2. Gibson MK, Forastiere AA (2006) Reassessment of the role of induction chemotherapy for head and neck cancer. Lancet Oncol 7(7):565–574
3. Bourhis J (2005) New approaches to enhance chemotherapy in SCCHN. Ann Oncol 16(Suppl 6):vi20–vi4
4. Hwang HS, Davis TW, Houghton JA, Kinsella TJ (2000) Radiosensitivity of thymidylate synthase-deficient human tumor cells is affected by progression through the G1 restriction point into S-phase: implications for fluoropyrimidine radiosensitization. Cancer Res 60(1):92–100
5. Ho YP, Au-Yeung SC, To KK (2003) Platinum-based anticancer agents: innovative design strategies and biological perspectives. Med Res Rev 23(5):633–655
6. Esaki T, Nakano S, Masumoto N, Fujishima H, Niho Y (1996) Schedule-dependent reversion of acquired cisplatin resistance by 5-fluorouracil in a newly established cisplatin-resistant HST-1 human squamous carcinoma cell line. Int J Cancer 65(4):479–484
7. Cho H, Imada T, Oshima T, Shiozawa M, Rino Y, Takanashi Y (2002) In-vitro effect of a combination of 5-fluorouracil (5-FU) and cisplatin (CDDP) on human gastric cancer cell lines: timing of cisplatin treatment. Gastric Cancer 5(1):43–46
8. Furukawa S, Tsukuda M, Mochimatsu I, Kokatsu T, Satoh H, Sakumoto M (1994) Modified combination chemotherapy of cisplatin and 5-fluorouracil in squamous cell carcinomas of the head and neck. Auris Nasus Larynx 21(3):181–185
9. Esaki T, Nakano S, Tatsumoto T, Kuroki-Migita M, Mitsugi K, Nakamura M et al (1992) Inhibition by 5-fluorouracil of cis-diamminedichloroplatinum(II)-induced DNA interstrand cross-link removal in a HST-1 human squamous carcinoma cell line. Cancer Res 52(23):6501–6506
10. Tanaka T, Masuda H, Naito M, Tamai H (2001) Pretreatment with 5-fluorouracil enhances cytotoxicity and retention of DNA-bound

platinum in a cisplatin resistant human ovarian cancer cell line. Anticancer Res 21(4A):2463–2469
11. Schutte B, Reynders MM, van Assche CL, Hupperets PS, Bosman FT, Blijham GH (1987) An improved method for the immunocytochemical detection of bromodeoxyuridine labeled nuclei using flow cytometry. Cytometry 8(4):372–376
12. Kawashima R, Haisa M, Kimura M, Takaoka M, Shirakawa Y, Takeda H et al (2004) Cyclin A correlates with the sensitivity of human cancer cells to cytotoxic effects of 5-FU. Int J Oncol 24(2):273–278
13. Robinson HM, Jones R, Walker M, Zachos G, Brown R, Cassidy J et al (2006) Chk1-dependent slowing of S-phase progression protects DT40 B-lymphoma cells against killing by the nucleoside analogue 5-fluorouracil. Oncogene 25(39):5359–5369
14. Takeda H, Haisa M, Naomoto Y, Kawashima R, Satomoto K, Yamatuji T et al (1999) Effect of 5-fluorouracil on cell cycle regulatory proteins in human colon cancer cell line. Jpn J Cancer Res 90(6):677–684
15. Mirjolet JF, Didelot C, Barberi-Heyob M, Merlin JL (2002) G(1)/S but not G(0)/G(1)cell fraction is related to 5-fluorouracil cytotoxicity. Cytometry 48(1):6–13
16. Angus SP, Wheeler LJ, Ranmal SA, Zhang X, Markey MP, Mathews CK et al (2002) Retinoblastoma tumor suppressor targets dNTP metabolism to regulate DNA replication. J Biol Chem 277(46):44376–44384
17. Wilson GD, Bentzen SM, Harari PM (2006) Biologic basis for combining drugs with radiation. Semin Radiat Oncol 16(1):2–9