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Inhibition of repair of carboplatin-induced DNA damage by 9-β-D-arabinofuranosyl-2-fluoroadenine in quiescent human lymphocytes

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Abstract

Previous studies including ours have demonstrated that DNA repair is one of the important targets of fludarabine. The aim of this study is to clarify a mechanistic interaction of carboplatin and F-ara-A, from the perspective of F-ara-A-mediated inhibition of DNA repair initiated by carboplatin. Using human quiescent lymphocytes, we focused on DNA repair, since these cells provide a model of dormant cells. To evaluate the carboplatin-induced DNA incision and its repair, we used the alkaline comet assay. When lymphocytes were incubated with carboplatin, a dose-dependent increase in the tail-moment was observed. Then, tail-moment decreased in proportion to the incubation period in fresh media and recovered to the control level at 4 h. DNA rejoining was completely inhibited by F-ara-A at 10 μM through 0 to 6 h after washing out of these drugs and this F-ara-A-induced inhibition was concentration-dependent. Cellular damage after drug exposure was evaluated with the induction of apoptosis as well as cytotoxic effect. Exposure to carboplatin alone did not induce any apparent cellular damage in quiescent lymphocytes. In contrast, a more than additive induction of apoptosis as well as an enhancement of cytotoxic action was observed in cells treated with a combination of carboplatin and F-ara-A. In the CEM cell line, there was no enhancement of the cytotoxic action of these drugs, despite the clear demonstration of an inhibitory effect on DNA repair. These results indicate that chemotherapy with carboplatin opened a new target for F-ara-A by initiating DNA repair in quiescent cells.

Keywords: DNA repair; Fludarabine; Carboplatin; Comet assay; Lymphoma; Salvage therapy

1. Introduction

The efficacy of carboplatin has been confirmed in patients with several kinds of solid cancers such as lung, ovary, cervical and urological cancer [1]. In contrast, the role of carboplatin for the patients with malignant lymphoma appears not to have been fully evaluated. Carboplatin has shown fair activity against lymphoid cell lines in vitro, compared to those against solid tumor cell lines. The IC_{50} value of carboplatin against representative lymphoid cell lines such as CESS, SKW6.4 and CCRF-CEM were 3.5 ± 0.4, 4.5 ± 2.1 and 13.7 ± 4.7 μM, respectively [2]. The mean value of IC_{50} against these cell lines was 7.4 ± 1.7 μM, while mean IC_{50} value against ovary, cervix, prostate and lung cancer cell line were 21.7 ± 7.2, 34.2 ± 10.6, 48.7 ± 18.8 and 106.5 ± 8.8 μM, respectively [2]. Clinical experience and published data on carboplatin-containing treatments have also been too limited to conclusively evaluate the role in patients with malignant lymphoma. Danieu reported that partial response was obtained in only two of 23 patients with diffuse large B-cell lymphoma by 350 mg/m^2 carboplatin i.v. infusion [3]. In contrast, Naito et al. reported a somewhat promising result of monotherapy. Two and 11 of 33 patients with lymphoma were evaluated as complete response and partial response following treatment with carboplatin i.v. infusion for 30–60 min at a dose range of 200–400 mg/m^2 [4], suggesting an expected potential of combined therapy with other types of anticancer agents. To develop new combina-

Abbreviations: F-ara-A, 9-β-D-arabinofuranosyl-2-fluoroadenine; IC_{50}, the concentration to inhibit 50% growth; CLL, chronic lymphocytic leukemia; F-ara-ATP, 9-β-D-arabinofuranosyl-2-fluoroadenine triphosphate; UDS, unscheduled DNA synthesis; 4-HC, 4-hydroperoxycyclophosphamide; NER, nucleotide excision repair; LDH, lactate dehydrogenase

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tional regimens that include carboplatin while offering a better prognosis for patients with lymphoma, it is important to clarify the mechanistic interaction(s) with other anticancer agents.

F-ara-A is a purine nucleoside analogue, which has been shown to be active against CLL and other indolent B-cell malignancies [5]. In contrast, F-ara-A has only modest activity as a single agent against aggressive lymphoma, ranging 10–15% [6], although it has been demonstrated that there was a tendency for higher values of deoxycytidine kinase, which is a key enzyme in accumulating F-ara-ATP, an active metabolite of F-ara-A, in more malignant histological subtypes of non-Hodgkin's lymphoma [7]. Recent insights have demonstrated that F-ara-A is a potent inhibitor of DNA repair at multiple steps. Those includes DNA polymerase ε, DNA ligase I, ribonucleotide diphosphate reductase [8]. Several lines of evidence have demonstrated that the inhibitory effect of F-ara-A on DNA repair, which was initiated by DNA-damaging agents, produced more than additive cytotoxic effects on both proliferating and quiescent cells. Lee et al. demonstrated that F-ara-A synergistically interacts with cisplatin in the proliferating colon cancer cell line Lovo [9]. Previously our studies have shown that combinational treatment of F-ara-A and 4-HC, an active form of cyclophosphamide, also produced synergistic cytotoxicity in normal and CLL lymphocytes [10,11]. Thus, inhibition of DNA repair by F-ara-A may open new chemotherapeutic targets in both the proliferating and quiescent tumor cell fractions.

Single-cell gel-electrophoresis (comet) assay was first introduced by Östling and Johanson [12]. It is a rapid, simple and sensitive technique for detecting DNA strand-break including DNA repair intermediates generated after removal of damaged DNA. We have previously demonstrated that comet assay is capable of evaluating a DNA repair process, which is affected by F-ara-A in normal and CLL lymphocytes [10].

The aim of this study is to clarify a mechanistic interaction of carboplatin and F-ara-A, from the perspective of F-ara-A-mediated inhibition of DNA repair initiated by carboplatin. We investigated carboplatin-induced DNA damage in the presence of F-ara-A. In major part of the present study, quiescent lymphocytes were employed as an experimental model for two reasons. First, these cells facilitate a focus in DNA repair and elucidate the action of F-ara-A on DNA repair by disregarding DNA replication. Second, these cells are a model of dormant cells, which participate in the resistance to S-phase specific agents such as carboplatin. We demonstrated that carboplatin induced UDS in normal human lymphocytes. The comet assay demonstrated that clinically achievable concentration of F-ara-A efficiently inhibits DNA repair induced by carboplatin. Furthermore, combination treatment produced more than additive induction of apoptosis as well as enhancement of cytotoxic effects on lymphocytes. By contrast, carboplatin alone did not produce any cytotoxic action in quiescent cells. In a proliferating cell line CEM, no apparent enhanced cytotoxic effect of combination treatment was observed, although an inhibitory effect of F-ara-A on DNA repair initiated by carboplatin was confirmed by comet assay. Our data suggest a mechanism-based rationale for treatment with carboplatin and F-ara-A in combination especially for resistant lymphoid malignancies, which contain dormant cells.

2. Materials and methods

2.1. Chemicals and reagents

Carboplatin was kindly supplied by Bristol Pharmaceuticals K.K. F-ara-A and hydroxyurea were purchased from Sigma-Aldrich. [Methyl-1′,2′-^{3}H] thymidine (123 Ci/mmol) was purchased from Amersham Biosciences. The fluorogenic substrate for caspase-3/-7, Ac-DEVD-MCA (acetyl-asp-glu-val-asp-[4-methyl-coumaryl-7-amide]) was purchased from Peptide Institute Inc.

2.2. Preparation of human lymphocytes from a healthy donor

Human lymphocytes from a healthy donor were used after informed consent was obtained. Whole blood was drawn into heparinized tubes layered over Ficoll-Paque™ PLUS (Amersham Biosciences) and centrifuged at 1500 rpm for 20 min. Normal lymphocytes were harvested from the interphase, washed twice with phosphate-buffered saline (PBS) and counted. The lymphocytes were resuspended at 2×10^6 cells/ml in RPMI 1640 supplemented with 10% heat-inactivated fetal calf serum (GIBCO, Invitrogen Corp.), 2 mM L-glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin (Nacalai Tesque). The cells were incubated in a 5% CO_2-humidified atmosphere in a culture bottle at 37 °C overnight to remove adherent cells.

2.3. Carboplatin-induced UDS

UDS during carboplatin-induced repair was evaluated by assessing the incorporation of tritiated thymidine. Normal lymphocytes (8×10^6 cells) were incubated with 2 mM hydroxyurea to suppress any DNA replication. After treatment with carboplatin, cells were washed twice and incubated in 2 ml of fresh medium consisting of RPMI 1640 and 10% dialyzed FBS (GIBCO, Invitrogen Corp.) with tritiated thymidine (20 μCi/sample) for 4 h. The lymphocytes were washed twice with ice-cold PBS and collected on a filter by aspiration. After treating with 10% trichloroacetic acid twice and washing with distilled water, the radioactivity retained on the filter was deter-

mined with a liquid scintiration counter, LSC-3500 (Aloka Co. Ltd.).

2.4. Quantitation of DNA incision by the alkaline single-cell gel-electrophoresis (comet) assay

To evaluate the DNA incision of each sample, we used the alkaline comet assay as described by Singh et al. [13] with slight modifications. For this assay, 5×10^4 cells after drug treatment were mixed with 75 μl of 0.5% low melting point agarose in PBS at 37 °C. The mixture was layered onto a frosted microscope slide glass (MATUNAMI) previously coated with 100 μl of 1.0% of normal agarose in PBS, followed by a top layer of 75 μl of 0.5% low melting point agarose. After solidification at 4 °C, the slides were left in a lysis solution (2.5 M NaCl, 10 mM Tris, 100 mM EDTA, 10% DMSO, and 1% Triton X-100, pH 10) at 4 °C for at least 1 h. The slides were then placed in electrophoresis buffer (1 mM EDTA, 300 mM NaOH, pH > 13) for 40 min at 4 °C to allow unwinding of the DNA. Electrophoresis was conducted for the next 40 min at 25 V (0.7 V/cm) with 300 mA for lymphocytes, and 15 min at 90 V (2.5 V/cm) with 500 mA for cell line. After electrophoresis, the slides were washed with neutralization buffer (0.4 M Tris, pH 7.5) and stained with 75 μl of 20 μg/ml ethidium bromide. Cells, in 100/treatment condition, were analyzed at 200× with an epifluorescence microscope (OLYMPUS) connected through a black and white charge-coupled device video camera to a computer-based image analysis system (Kinetic Imaging Komet system, version 4.0). Cellular responses to DNA damage were expressed as the ''tail moment'', which combines a measurement of the length of the DNA migration and the relative DNA content therein [14].

2.5. Measurement of the intracellular F-ara-ATP concentration in lymphocytes

Intracellular accumulation of F-ara-ATP was quantified using HPLC system [15]. After treatment with F-ara-A, cells were washed twice with ice-cold PBS and harvested by centrifugation. Cell pellet was lysed with 30 μl of 15% perchloric acid for 15 min on an ice bath and acid soluble material was collected by centrifugation, followed by an addition of 1N KOH. The neutralized supernatant was injected onto an anion-exchange column, TSK gel DEAE-2 SW (250× 4.6 nm i.d., TOSOH Corp.). The HPLC system consisted of a pump CCPM-II (TOSOH Corp.), an autosampler AS-8020 (TOSOH Corp.), an in-line degasser SD-8022 (TOSOH Corp.). The chromatography was controlled and analyzed by a personal computer equipped with the software LC-8020 (TOSOH Corp.). Elution was performed isocratically with 0.06 M Na_2HPO_4 (pH 6.9) 20% acetonitrile at a constant flow rate of 0.7 ml/min and an ambient temperature. F-ara-ATP was monitored at 261 nm. The concentration of intracellular F-ara-ATP was calculated according to the calibration curve of external authentic standard.

2.6. Quantitation of apoptotic cell death induced by drug treatment

Caspase-3/-7 enzyme activity of each sample was measured by Dimmeler's method with slight modification [16]. After incubation, cells were washed and lysed in buffer (1% Triton X-100, 0.32 M sucrose, 5 mM EDTA, 1 mM phenylmethylsulfonyl fluoride (PMSF), 1 mg/ml aprotinin, 1 mg/ml leupeptin, 2 mM DL-dithiothreitol (DTT), 10 mM Tris–HCl, pH 8) for 15 min at 4 °C, followed by centrifugation (20,000 × *g*, 5 min). The caspase-3/-7 activity was detected by measuring the fluorogenic MCA liberated from the caspase-3-like substrate, Ac-DEVD-MCA, in assay buffer (100 mM Hepes, 10% sucrose, 0.1% 3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulfonate, pH 7.5, 1 mM PMSF, 1 mg/ml aprotinin, 1 mg/ml leupeptin, 2 mM DTT) using an excitation wavelength of 380 nm and an emission wavelength of 460 nm. The activity of caspase was expressed as fold increment of lysate from untreated control cells.

Sub-G_1 fraction of ethanol-fixed cells was quantified by flow cytometry. After incubation with drug, the lymphocytes were collected by centrifugation and fixed with 70% ethanol for 30 min on ice, then incubated with 3 μg/ml RNase at 37 °C for 20 min. After centrifugation, propidium iodide (PI) in 0.5% Tween-20 was added and analyzed with a FACScan flow cytometry (Becton Dickinson). Data acquisition and analysis were performed by CellQuest program (Becton Dickinson). The percentage of sub-G_1 population in each sample was calculated and considered as apoptotic. Untreated cells were used as a control.

2.7. Measurement of cytotoxic effect

Viable cells after drug treatment were counted by trypan blue dye-exclusion method. Viable cell count was expressed as 100% of untreated cells. LDH activity was used as a marker of cellular injury. Briefly, normal lymphocytes (2.0×10^5 cells/well) were seeded on a 96-well plate. After 24 h incubation, released LDH activity in the medium supernatant ws determined by commercially available kit according to manufacture's instruction (Promega). Percent cytotoxicity was calculated as follows: cytotoxicity (%) = (experimental value − spontaneous LDH release)/maximum LDH release.

2.8. Statistical analysis

Statistical analysis in Figs. 6–8 were performed with StatView 5.0 (SAS Institute Inc.). The difference of each group was evaluated by ANOVA followed by post-hoc test of Bonferroni/Dunn method.

3. Results

3.1. Induction of UDS by carboplatin-induced DNA damage

Our first objective is to determine whether carboplatin has the ability to initiate DNA repair in human quiescent lymphocytes. To evaluate the UDS initiated by carboplatin, normal human lymphocytes were incubated with various concentration of carboplatin for 90 min, and further incubated in the fresh media with tritiated thymidine for 4 h (Fig. 1). After induction of DNA damage, UDS increased in a concentration-dependent manner of carboplatin and did not reach a plateau at least up to 150 μM.

3.2. Quantitation of DNA incision induced by carboplatin by comet assay

To determine carboplatin-induced DNA incision, the comet assay was introduced. When quiescent human lymphocytes were incubated for 90 min in the presence of various concentration of carboplatin up to 150 μM, a dose-dependent increase in the tail moment was observed (Fig. 2A). The tail moment appeared to show a linear correlation with dose in a range, from 35 to 150 μM carboplatin, which was consistent with UDS shown in Fig. 1. When cells were incubated in the presence of 150 μM carboplatin up to 90 min, a time-dependent increase in the tail moment was observed (Fig. 2B). When the incubation period was extended over 90 min, the tail moment once decreased then reached a plateau. This plateau was sustained at least 6 h incubation. On the basis of these results, evaluation of DNA repair in the subsequent studies was initiated by incubation with 150 μM carboplatin for 90 min. There were few, if any, increments of tail moment observed when cells were treated with carboplatin at 4 °C to suppress any biological process associated with DNA repair (data not shown).

To address DNA repair initiated by carboplatin, lymphocytes were first incubated in the presence of 150 μM carboplatin, further incubated in fresh media. This time course of DNA incision was evaluated by comet assay (Fig. 3). It was found that the tail moment was greatest at the end of the incubation with carboplatin. The tail moment decreased in proportion to the incubation period in fresh media. The rapid decrease in tail moment values repre-

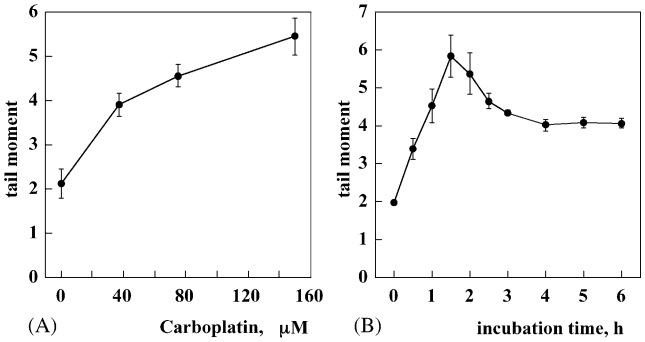

Fig. 2. Induction of DNA incision by carboplatin. (A) Normal lymphocytes from a healthy donor were incubated in the presence of the indicated concentration of carboplatin. DNA incision induced by carboplatin was determined by the comet assay as described in Section 2 (means; bars, S.D., $n = 3$). (B) Cells were also incubated in the presence of 150 μM carboplatin for the indicated periods. At the end of incubation, carboplatin-induced DNA incision was determined by the comet assay.

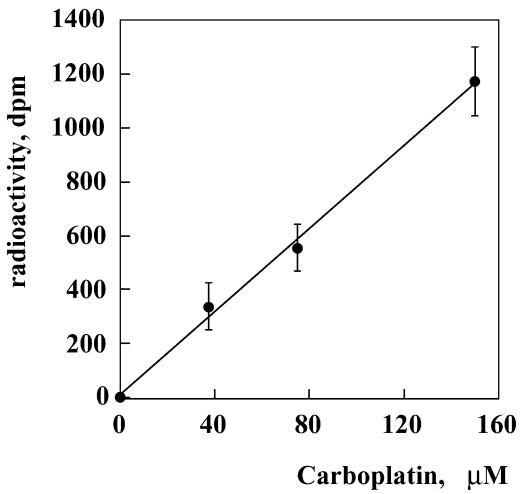

Fig. 1. Carboplatin-induced DNA repair in human normal lymphocytes. Normal lymphocytes were incubated in the presence of carboplatin at the indicated concentration for 90 min. The cells were immediately followed by washing twice and subsequently incubating in 2 ml of fresh medium with tritiated thymidine (20 μCi/sample) for 4 h. Then, the radioactivity of incorporated tritiated thymidine was measured by filter-binding assay. (means; bars, S.D., $n = 3$).

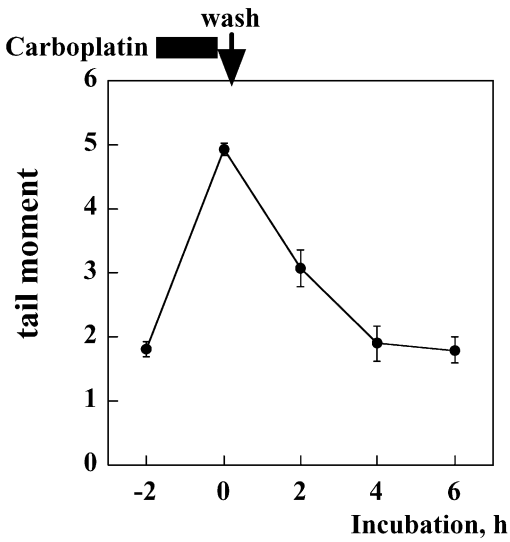

Fig. 3. DNA repair after carboplatin-induced DNA damage evaluated by comet assay. Normal lymphocytes were incubated with 150 μM carboplatin for 90 min to induce DNA damage and then washed. Cells were further incubated in the fresh medium to allow DNA repair, and the tail moment was assayed at the indicated time point. The tail moment at 0 h indicates carboplatin-induced DNA incision. The decrease in tail moment indicates rejoining of the DNA repair process. (means; bars, S.D., $n = 3$).

sented the rate of completion of the repair process after DNA ligation. The rate appeared to decrease to half the peak value by 2 h. After 4 h incubation in drug-free media, the tail moment recovered to the control level. Collectively, DNA repair induced by carboplatin takes place rapidly after DNA damage and is completed till 4 h after removal of carboplatin in normal lymphocytes. Based on these results, we quantitated the kinetics of repair till 6 h after induction of DNA damage.

3.3. F-ara-A inhibits carboplatin-induced DNA repair

Our previous studies have shown that F-ara-A is capable of inhibiting DNA repair induced by 4-HC in normal and CLL lymphocytes [10,11]. However, it is not clear whether F-ara-A inhibits DNA repair initiated by carboplatin in human lymphocytes. To address this issue, lymphocytes were exposed to escalating concentrations of F-ara-A for 30 min and further incubated for 90 min after the addition of 150 μM carboplatin. After drug treatment, cells were washed extensively and further incubated in fresh media till 6 h to allow repair. DNA incision in these cells was evaluated by comet assay (Fig. 4A). The rate of completion of repair after washout drugs was found to be inversely proportional of the concentration of F-ara-A. It was found that DNA rejoining was completely inhibited by F-ara-A at 10 μM. In this experiment, an increase in the tail-moment value in the presence of F-ara-A and carboplatin represented a combination of on-going incision and inhibition of the ability of cells to complete the repair process. Immediately after incubation with F-ara-A and carboplatin, there were few differences, if any, in DNA incision according to the concentration of F-ara-A administered. To further elucidate the concentration dependency of F-ara-A on the inhibitory effect on the repair of carboplatin-induced DNA damage, DNA incision was quantitated after 4 h-incubation in fresh media (Fig. 4B). The inhibitory effect showed a linear relationship to the concentration of F-ara-A up to 3 μM then appeared to reach a plateau.

3.4. Accumulation and sustained retention of F-ara-ATP in human lymphocytes

The enzymatic conversion by deoxycytidine kinase from F-ara-A to F-ara-ATP prior to incorporation into DNA has been shown to be indispensable for activation [8]. To address whether the inhibitory effect of F-ara-A on DNA repair observed above is mediated by the mechanisms of F-ara-A action, we quantitated intracellular accumulation of F-ara-ATP in lymphocytes. Cells were incubated for 2 h in the presence of various concentrations of F-ara-A and then intracellular F-ara-ATP was quantified by HPLC. The result indicated that intracellular F-ara-ATP increased in a concentration-dependent manner of F-ara-A (Fig. 5A).

DNA rejoining was efficiently inhibited for 6 h even after washout of F-ara-A from media as shown in Fig. 4A. To confirm the retention of intracellular F-ara-ATP, lymphocytes were incubated with 10 μM F-ara-A for 2 h and further incubated in fresh medium up to 6 h (Fig. 5B). At the end of incubation with F-ara-A, the level of intracellular F-ara-ATP reached 65 ± 4 pmol/10^7 cells. Six hours after washout of F-ara-A from medium, the intracellular F-ara-ATP remained 83 ± 5% of the level at 0 h. These results strongly suggested that F-ara-A was able to inhibit DNA repair efficiently due to the retention of F-ara-ATP even after disappearance of the prodrug from medium.

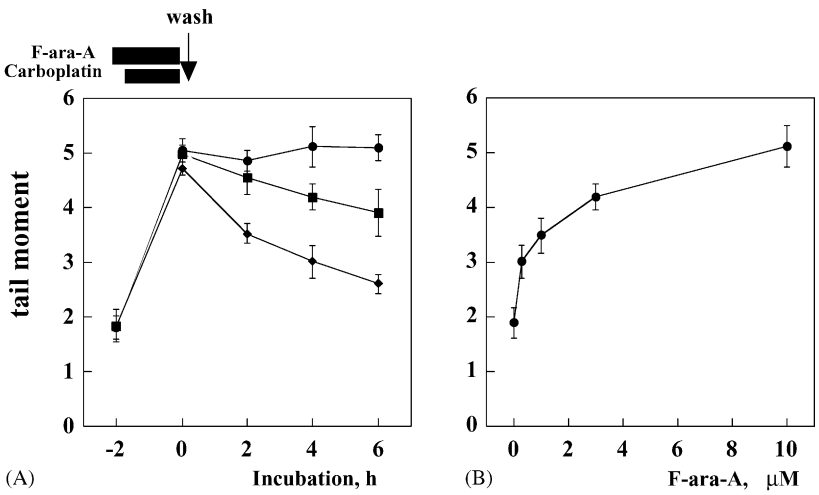

Fig. 4. Inhibitory effects of carboplatin-induced DNA repair by F-ara-A. (A) Normal lymphocytes were preincubated with the indicated concentration of F-ara-A (0.3 μM, closed diamond; 3 μM, closed square and 10 μM, closed circle) for 30 min, subsequently coincubated with 150 μM carboplatin for 90 min. Then, cells were washed and transferred to fresh medium. The tail-moment was assayed to evaluate the repair process (means; bars, S.D., $n = 3$). (B) Cells were incubated in the presence of the indicated concentration of F-ara-A for 30 min, and further incubated for 90 min after addition of 150 μM carboplatin. After wash out of drugs, cells were incubated in fresh medium for 4 h and the tail moment was determined.

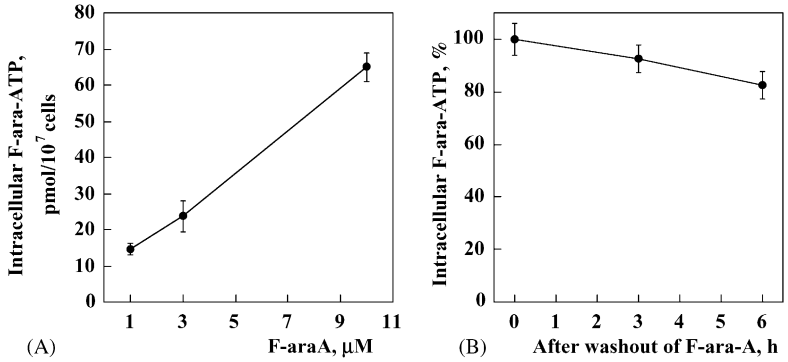

Fig. 5. Intracellular concentration of F-ara-ATP. (A) Human normal lymphocytes were incubated with the indicated concentration of F-ara-A for 2 h. At the end of incubation, intracellular F-ara-ATP was determined by HPLC (pmol/10^7 cells: mean, S.D., $n = 3$). At 2 h incubation of 10 μM F-ara-A, intracellular F-ara-ATP was 65 ± 4 pmol/10^7 cells. (B) After 2 h of incubation with 10 μM F-ara-A, cells were washed out and then resuspended into fresh medium for the indicated periods. At the end of each period, intracellular F-ara-ATP concentration was determined. (means; bars, S.D., $n = 3$).

3.5. More than additive induction of apoptosis by simultaneous exposure to F-ara-A and carboplatin in combination

Next, we examined whether F-ara-A-mediated inhibition of repair following carboplatin-induced DNA damage was associated with apoptosis in quiescent lymphocytes. We first quantitated the activity of caspase-3/-7, a hallmark of apoptosis, after drug treatment. Cells were pulse-challenged with 10 μM F-ara-A and 150 μM carboplatin as shown in Fig. 4A, and further incubated in fresh media for 24 h. The activation of caspase-3/-7 was not observed under this experimental condition in combinationally treated cells (data not shown), although carboplatin-induced DNA incision persisted for at least 6 h (Fig. 4A). Thus, cells were incubated for 30 min with or without 10 μM F-ara-A, followed by continuous incubation with 150 μM carboplatin for up to 24 h (Fig. 6A). The activity of caspase-3/-7 remained unchanged in cells treated with carboplatin alone, confirming that this S-phase specific agent had no cytotoxic effect against quiescent cells. In cells treated with F-ara-A as a single agent, the moderate activation of caspase-3/-7 was observed in a time-dependent manner. The caspase-3/-7 activity at 24 h incubation was 1.76 ± 0.72-fold of the control value. The mechanisms of F-ara-A to induce apoptosis in quiescent lymphocytes have shown to be an inhibition of RNA synthesis by incorporation into RNA [17] or the direct association of F-ara-ATP with apaf-1 and cytochrome-*c*, which further activates caspase-3/-7 [18]. In contrast, after 24 h incubation with F-ara-A and carboplatin, caspase-3/-7 activity was found to be 2.78 ± 0.35-fold of the control value, indicating that the combinational effect was significantly more than additive as compared to the effects observed in those treated with each drug alone (Fig. 6A). We also examined a sub-G_1 fraction of ethanol-fixed cells after incubation with drugs under the same conditions as above (Fig. 6B). An increment in the sub-G_1 fraction was not observed in cells continuously exposed to carboplatin alone (3.1 ± 0.4% of gated events), and small but substantial increment in sub-G_1 fraction in cells treated with F-ara-A as a single agent (5.6 ± 0.8% of gated events). It was confirmed that an increment of sub-G_1 fraction of combinationally treated cells was significantly more than additive (18.1 ± 0.7% of gated events). We conclude that augmented induction of apoptosis was associated with the F-ara-A-mediated inhibition of repair following carboplatin-induced DNA damage.

3.6. Enhanced cytotoxic effect by simultaneous exposure of human lymphocytes to a combination of F-ara-A and carboplatin

To further confirm cytotoxic action by combination treatment with F-ara-A and carboplatin, we also counted viable cells by trypan blue dye-exclusion method (Fig. 7A). Lymphocytes were treated with F-ara-A, carboplatin, or a combination of these two drugs for 24 h as shown in Fig. 6. Loss of viable cells after treatment of carboplatin alone, F-ara-A alone or a combination of these drugs was 5.1, 12.3 and 29% of control, respectively. We also conducted LDH releasing assay using the same treatments as above (Fig. 7B). Percent cytotoxicities after treatment with carboplatin alone, F-ara-A alone or a combination of these drugs were 4.7 ± 0.8, 10.7 ± 1.1 and 27.0 ± 2.3, respectively. The enhanced cytotoxic effect of combination treatment was observed.

3.7. No apparent enhancement in cytotoxic effect of F-ara-A and carboplatin in proliferating cells

Finally, we extended our study to a CEM cell line, a representative lympoblastic leukemia cell line, to clarify the mechanistic interaction of F-ara-A and carboplatin in proliferating cells. CEM cells were treated with an increasing concentration of F-ara-A for 30 min, followed by 40 μM carboplatin for 90 min. Under these conditions, a dose-dependent inhibitory effect of F-ara-A on DNA repair

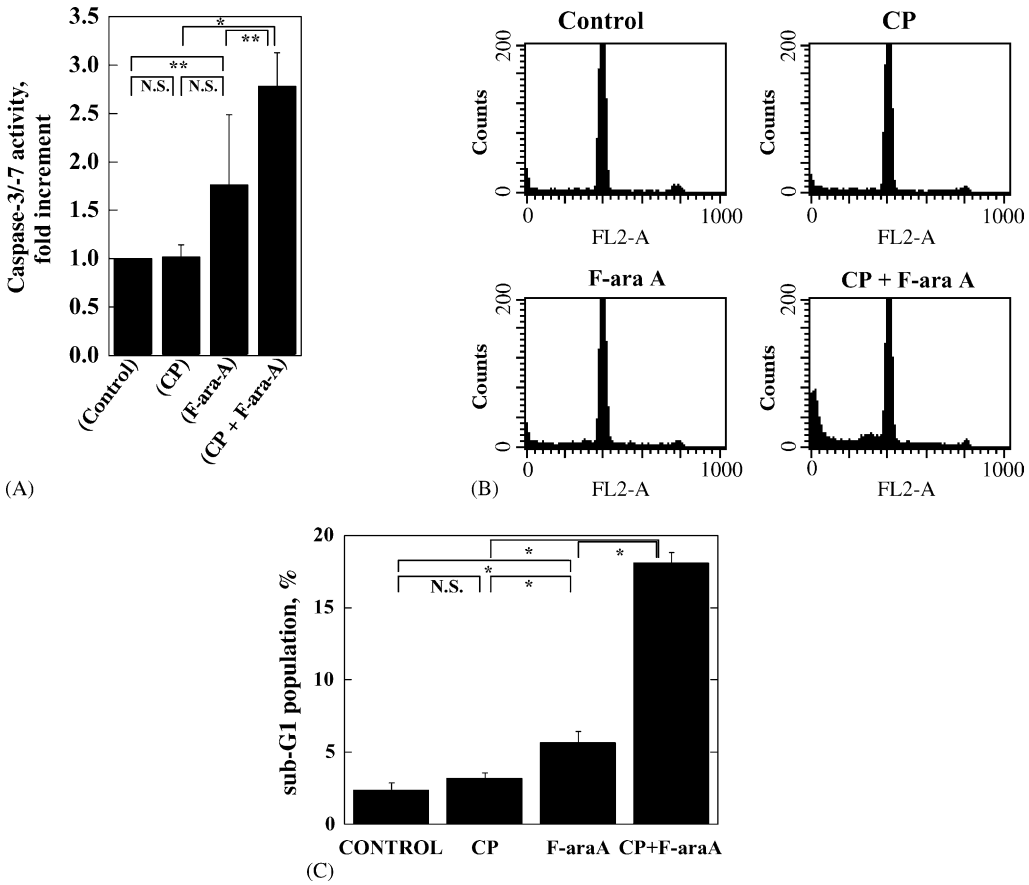

Fig. 6. Enhanced induction of apoptosis in cells treated with a combination of F-ara-A and carboplatin. Normal lymphocytes (7.5×10^6 cells) were incubated for 30 min with or without 10 μM F-ara-A, followed by continuous incubation with 150 μM carboplatin for 24 h (CP). After incubation, the caspase-3/-7 enzyme activity was determined to evaluate the induction of apoptosis by combination treatment with these drugs (A). Cells in the sub-G1 population were also determined after PI staining. A representative histogram of cells (B). Graphic presentation of sub-G1 populations of a representative result in three independent experiments (C) (means; bars, S.D., $n = 3$) ($^{*}P < 0.01$, $^{**}P < 0.05$, N.S.: not significant).

initiated by carboplatin was confirmed (Fig. 8A). We evaluated viable cell count, since we failed to detect typical apoptotic features. The growth inhibitory effect of carboplatin, F-ara-A, or a combination after 24 h continuous treatment was 23, 62 and 92%, respectively, indicating that the enhanced combinational effect of F-ara-A and carboplatin was not seen (Fig. 8B).

4. Discussion

Our previous studies demonstrated that DNA repair initiated by UV or 4-HC in quiescent lymphocytes allowed F-ara-AMP incorporation into the repair patch, an action that was associated with the inhibition of the repair process. Such interaction of nucleoside analogue and DNA

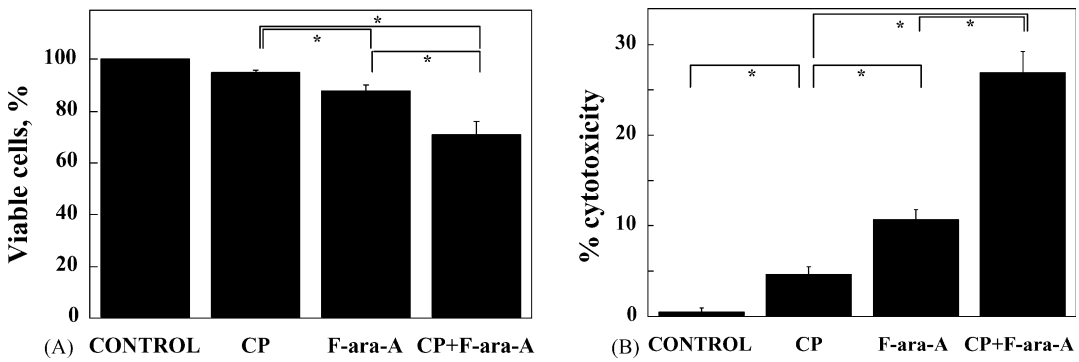

Fig. 7. Enhanced cytotoxic effect in domant lymphocytes by combination treatment with carboplatin and F-ara-A. Normal lymphocytes were incubated for 30 min with or without 10 μM F-ara-A, followed by 24 h continuous incubation with 150 μM carboplatin (CP). After 24 h of coincubation, viable cells were counted by trypan blue dye-exclusion method (A), and dead cells were evaluated by LDH-release assay (B) (means; bars, S.D., $n = 3$) ($^{*}P < 0.01$).

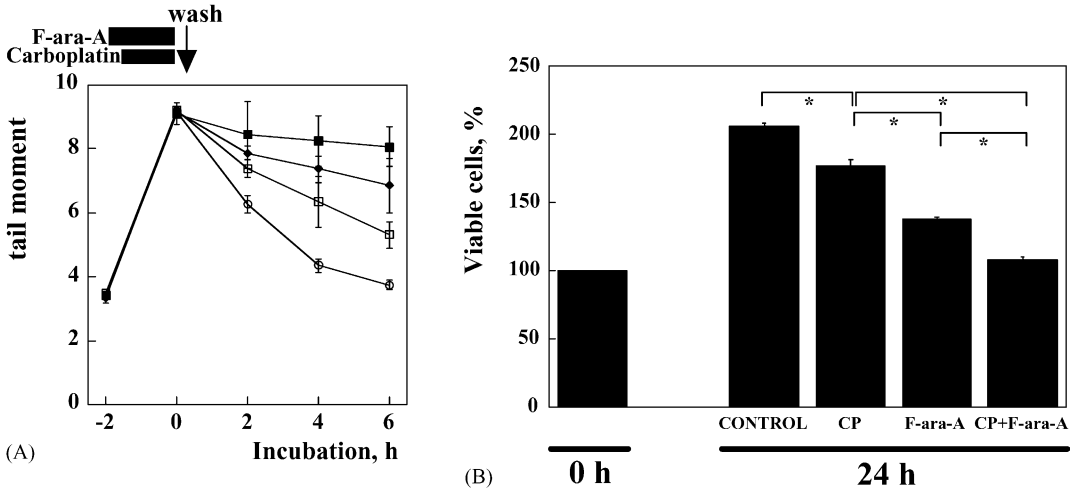

Fig. 8. No apparent enhanced effect of combinational treatment with F-ara-A and carboplatin in a proliferating cell line. (A) Inhibitory effect of F-ara-A on carboplatin-initiated DNA repair. CEM cells were preincubated without (open circle) or with the indicated concentration of F-ara-A (0.3 μM, open square; 1 μM, closed diamond and 3 μM, closed square) for 30 min, subsequently coincubated with 40 μM carboplatin (CP) for 90 min. Then, cells were washed and transferred to fresh medium. The tail moment was assayed to evaluate the repair process. (means; bars, S.D., $n = 3$). (B) No apparent enhanced effect of combined treatment with F-ara-A and carboplatin on growth inhibitory effect. CEM cells were incubated for 30 min with or without 3 μM F-ara-A, followed by 24 h continuous incubation with 40 μM CP. After 24 h of coincubation, viable cells were counted by trypan blue dye-exclusion method as 100% of the cell count at 0 h. (means; bars, S.D., $n = 3$) ($^{*}P < 0.01$).

damage enhanced the cytotoxic action [10]. In a series of experiments including the present study, we have focused on quiescent lymphocytes without replicative DNA synthesis for two reasons: (1) to clarify the mechanistic interaction of DNA-damaging agents and F-ara-A from the perspective of inhibiting DNA repair; (2) to evaluate whether this mechanistic interaction is effective against dormant cells, which may contribute to drug resistance in the tumor, since these cells are free from targets of S-phase specific anticancer agents. The present study extended this approach to carboplatin, since the role of carboplatin in the treatment of lymphoid malignancies has not been yet clarified [19].

Carboplatin has been reported to form adducts with isolated DNA as follows; 58% *cis*-$Pt(NH_3)_2$d(pGpG) di-adducts, 11% *cis*-$Pt(NH_3)_2$d(pApG): di-adducts, 9% *cis*-$Pt(NH_3)_2$d$(GMP)_2$ di-adducts, which are the same DNA adducts as those induced by cisplatin [20], which have been shown to be eliminated by NER [21]. Thus, it is likely that NER plays an important role in eliminating carboplatin-induced DNA adducts. Single-strand breaks are generated at excision steps of NER, which are detected by the comet assay (Fig. 2A and B). To support this, we confirmed that few increments of tail moment were observed, when cells were treated with carboplatin at 4 °C to suppress any biological process associated with DNA repair (data not shown).

Both the incorporation of thymidine (Fig. 1) and the tail moment after treatment with carboplatin (Fig. 2A) confirmed that quiescent lymphocytes were proficient in repairing DNA damage initiated by carboplatin up to 150 μM. When carboplatin was administered at a dose range of 75–450 mg/m^2, the peak concentration ranged from 40 to 230 μM [22], which was similar to the concentration range shown in Fig. 2A. The steep decrease in tail moment during the subsequent repair period in fresh media suggested that ligation of repair patch had occurred after washout of carboplatin (Fig. 3). This kinetics shown by the comet assay is totally consistent with those demonstrated by other methods for evaluating overall genome repair of cisplatin-damaged DNA [9]. In conjunction with these previous reports and our observation, it is suggested that the repair process of platinum-compound-induced DNA damage was generally completed with 6 h.

We demonstrated that F-ara-A inhibits repair of carboplatin-induced DNA damage, as indicated by the decreased rate of repair after treatment with carboplatin (Fig. 4). In Fig. 4A, there was no difference observed in the tail moment at 0 h in the presence or absence of F-ara-A, which mainly represents incision steps of excision repair. Thus, F-ara-A seems to inhibit mainly the repair synthesis and the ligation steps. This suggestion is consistent with the previous study demonstrating that F-ara-A inhibits DNA ligation and DNA chain elongation in vitro [23,24].

Although the inhibitory effect of F-ara-A on DNA repair was found to be nearly complete at 10 μM (Fig. 4A), this inhibitory effect appeared to reach a plateau at 3 μM (Fig. 4B). It has been demonstrated that a 30-min infusion of 25–30 mg/m^2 F-ara-A to patients produced 2–7 μM F-ara-A concentrations in the plasma [25], indicating that the standard clinical dose of F-ara-A would be expected to inhibit DNA repair in patients.

The lack of a linear correlation between the inhibitory effect on DNA repair (Fig. 4B) and the intracellular accumulation of F-ara-ATP (Fig. 5A) might indicate that F-ara-A targeted multiple molecules engaged in DNA

repair with different affinities in lymphocytes. It has been demonstrated that those molecules include DNA polymerase ε and DNA ligase I. Kamiya et al. demonstrated that the 3′ → 5′-exonuclease activity of DNA polymerase ε bound to 3′-F-ara-AMP-terminated DNA with high affinity (K_m = 7 pM), and in turn, inactivated the exonuclease activity [26]. The concentration of F-ara-ATP required to inhibit the polymerase activity of DNA polymerase ε by 50% has been reported to be 1.3 μM on DNA primer extension assay [23]. F-ara-ATP also inhibits DNA ligase I by a dual mode [24]. Free F-ara-ATP at 80 μM inhibits activity of DNA ligase I by more than 90%. In addition, in vitro assay for ligation of single-strand DNA breaks has demonstrated that the incorporated F-ara-AMP at the 3′-position of DNA inhibited ligase activity, although the affinity of DNA ligase I in this lesion has not been demonstrated [24]. Further studies will be needed to elucidate the target, which is most critical for inhibiting DNA repair initiated by carboplatin in human lymphocytes.

We demonstrated that although intracellular F-ara-ATP was found to be stably retained in cells at least for 6 h in normal lymphocytes (Fig. 5B), the carboplatin-initiated repair occurs rapidly and is completed within 4 h (Fig. 3). We previously demonstrated that CLL lymphocytes treated with F-ara-A after completion of 4-HC-induced DNA repair did not form any comets [10]. These findings indicated that the mechanistic interaction of F-ara-A and carboplatin is schedule-dependent and may be more effective when F-ara-A precedes carboplatin treatment. In FPA regimen, which F-ara-A-mediated inhibition of DNA repair was incorporated, patients first received cisplatin 25 mg/m^2 as a continuous intravenous infusion over 24 h daily for 4 days, then received F-ara-A 30 mg/m^2 and ara-C 500 mg/m^2 on days 3 and 4. We speculated that the administration of F-ara-A and ara-C prior to cisplatin would be effective, from a mechanistic perspective.

Although DNA ligation was completely inhibited in cells pulse-treated with 10 μM F-ara-A and 150 μM carboplatin (Fig. 4A), this inhibition was not translated into major cell death (data not shown). In contrast, continuous exposure to carboplatin and F-ara-A showed enhanced cytotoxic action (Fig. 7). Lymphocytes treated with carboplatin for 24 h retained viability (Figs. 6 and 7), thus, it was suggested that lymphocytes were able to accomplish DNA repair during 24 h continuous exposure to carboplatin. As data to support this suggestion, Fig. 2B shows that DNA incision reached a plateau after 3–6 h incubation with carboplatin (Fig. 2B), indicating that on-going induction of DNA damage and repair reached an equilibrium. It is likely that F-ara-A was incorporated into the repair patch and, in turn, inhibited DNA repair. Therefore, F-ara-AMP incorporation and DNA incision accumulated in a time-dependent manner in continuously treated-cells that induced major cellular damage, but these lesions might be insufficient in pulse-treated cells (Fig. 7). We tried to quantitate the F-ara-A-mediated inhibition of DNA repair in cells continuously exposed to carboplatin and F-ara-A by comet assay. However, it was impossible to discriminate DNA incision derived from repair intermediate and other DNA strand-breaks such as DNA internucleosomal fragmentation associated with apoptosis. Whether the accumulation of unrepaired DNA or incorporation of F-ara-AMP into DNA is essential for initiating apoptosis in quiescent lymphocytes awaits further studies.

In the present study, the effect of F-ara-A and carboplatin in combined treatment was not fully evaluated. Although we conducted an isobologram analysis as described by Steel and Peckham, the cytotoxic action of carboplatin did not reach the IC_{50} value in the presence of a clinically relevant concentration [27]. Thus, the combined effect produced by F-ara-A and carboplatin was described as ''enhancement''. Despite a clear inhibitory effect of F-ara-A on carboplatin-initiated DNA repair on a proliferating CEM cell line, we failed to demonstrate any augmentation of cytotoxic effect (Fig. 8). Since both F-ara-A and carboplatin monotherapy elicited potent cytotoxic action; action due to a mechanistic interaction of these drugs might be inconspicuous under this condition. Otherwise, the discrepancy between the mechanistic interaction and growth inhibitory effect was specific to CEM. Supporting this possibility, Yang et al. demonstrated a synergistic effect of F-ara-A and cisplatin in the colorectal cancer cell line LoVo [9]. To elucidate the therapeutic potency in lymphoma cells, it will be necessary to compare the biochemical and cytotoxic action of this combination in a panel of lymphoma cell lines showing various degrees of chemosensitivity and proliferative activity.

In summary, we demonstrated F-ara-A inhibits DNA repair initiated by carboplatin treatment in human lymphocytes. This model warrants further clinical investigation to evaluate whether combination treatment with F-ara-A and carboplatin is effective especially in refractory and resistant lymphoma.

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