A Study of Long-Term Metabolism of Nucleic Acid in the Repair Process of Cancer Cells with Cis-diamminedichloroplatinum (II)

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SUMMARY : CDDP is one of the anticancer drugs in which the presence of the repair mechanism has been pointed out. Thus, using established pulmonary cancer cells, we evaluated the metabolism of DNA and RNA in damaged cells treated with CDDP (0.1, 1.0, 10 μg/ml) by means of ^{3}H-TdR・^{3}H-UR incorporation assay for 15 days referring to the growth curve and colony forming assay. ^{3}H-TdR uptake inhibition rate in CDDP treatment was higher than ^{3}H-UR uptake inhibition rate and showed good correlation with the inhibition rate of colony formation. In order to evaluate the activity of metabolism of DNA and RNA, the experimental period was divided into two parts : an early stage (2-10 days) and a later stage (7-15 days), and changing phases of uptake of ^{3}H-TdR and ^{3}H-UR was estimated as an increased rate. In the early stage, cells treated CDDP at lower concentrations tended to show more active DNA and RNA metabolism, especially RNA. In the later stage, cells with 0.1 μg/ml CDDP which had shown only slight growth inhibition in the early stage revealed lower activity than that in the early stage. Cells with 1.0 μg/ml CDDP which showed regrowth after 7 days, and cells with 10 μg/ml CDDP which were not recognized with proliferation were observed with more active metabolism of RNA than that in the early stage. Those results indicated that metabolism of RNA is closely associated with the cellular repair process by CDDP.

INTRODUCTION

At present, cis-diamminedichlorolplatinum (II) (CDDP) plays a major role in chemotherapy for various solid cancers. Especially, combination therapy of CDDP and Bleomycin (BLM) is highly effective, so that the response rate is 70% in cases of pulmonary squamous cell cancer and 79% in cases of cancer of neck and head[1,2]. However, KURITA et al.[3] reported that findings in investigations of tissues in combination therapy of CDDP and pepleomycin for cancer of neck and head showed that clinical effects (tumor reduction) did not always correspond to histological effects and poor prognosis was seen in cases that had high cellular degeneration and increased nuclear atypia in remaining cancer cells after chemotherapy. In the actual clinical field, we have

experienced many cases that obtained effective clinical outcomes at an early stage but were eventually recognized as recurrence and metastasis after that.

Thus, when regrowth of cancer cells in clinically effective cases is considered, it is necessary to take into consideration the presence of the repair mechanism of damaged cells by the anticancer drugs. Although the mechanism is not recognized in all anticancer drugs, the presence of the repair mechanism with CDDP and BLM is generally pointed out and many studies on it have been conducted[4]-[10]. Thus, we consider the necessity of effective drug administration for the purpose of inhibition of repair ability as maintenance therapy after the treatment by anticancer drugs. This time, we studied on this matter from the aspect of long-term metabolism of nucleic acid and conducted a basic experiment in which would be used DNA or RNA synthesis inhibitor as the blocker of repair using ^{3}H incorporation assay.

MATERIALS AND METHODS

1. Cells

The human cell line PC 1 of pulmonary squamous cell cancer which grow as monolayers (kindly supplied by Dr. Y. HAYATA of Tokyo Medical College, Japan) were grown in RPMI 1640 medium (GIBCO) supplemented with 10% fetal bovine serum (FBS, GIBCO). During the experiment, medium was exchanged once in every third day.

2. Anticancer drugs and reagent

CDDP (Provided by Nippon Kayaku Co., Ltd. Japan) was added with a final concentration of $0.1 \mu g/ml$, $1.0 \mu g/ml$ or $10 \mu g/ml$ at 24 hours after plating in dish. The exposure time was determined to be 2 hours, and the cultured cells were subsequently washed by PBS ($-$) and fresh medium was exchanged. Additionally, Mitomycin C (MMC, provided by Kyowa Hakko Co., Ltd. Japan) and Adriamycin (ADM, provided by Kyowa Hakko Co., Ltd. Japan), with concentration at which similar cell growth kinetics to CDDP $10 \mu g/ml$ were demonstrated, were investigated. ^{3}H-thymidine (^{3}H-TdR, Amersham international

plc. specific activity : 20 Ci/nmol) and ^{3}H-unidine (^{3}H-UR, Amersham international plc. specific activity : 20.7 Ci/nmol) were adjusted to $1 \mu Ci/ml$ by diluting RPMI 1640 supplemented with 10% FBS and then stored at 4℃.

3. Growth curve

5×10^4 cells/ml was plated in a 36 mm dish, and CDDP at each concentration was added after 24 hours and exposed with the cells for 2 hours. Subsequently, the growth curve for 15 days was studied. Viable cells were counted using Trypan Blue dye exclusion test.

4. Colony forming assay

For investigation of colony formation, a flat bottom microplate with 96 wells (CORNING 25870) was used. Medium 0.1 ml containing 3×10^2 cells was placed in each well. Twenty-four hours later, CDDP with each concentration was added, and 0.1 ml of fresh medium was then exchanged after wash. The cells were stained by Giemsa stain at days 7 and 15 in order to measure small colonies of more than 8 cells under the microscope [11]. The inhibition rate of colony formation was calculated by (1-number of colonies of sample/number of non-treated cells (control)) $\times 100$.

5. ^{3}H-TdR and ^{3}H-UR incorporation assay

The experiment was conducted under the same condition for colony formation, that is, 0.1 ml of $1.0 \mu Ci/ml$ ^{3}H-TdR and ^{3}H-UR was added in 0.1 ml sample medium in a flat-bottom microplate with 96 wells at 2, 5, 10 and 15 days, and labeling time of 24 hours was then taken. Following the labeling, at tached cells were treated using 0.25% typsin. Subsequently, the cells were treated with 5% TCA for 30 minutes at 4 ℃. Following that, the cancer cells harvested to a paper (LM 101-10. Lab. Science) using Automatic cell harvester (LABOMASH. Labo. science), were again washed twice by 5% TCA and were then dried at room temperature. In order to calculate intracellular uptake of ^{3}H-TdR and ^{3}H-UR by cpm using a fluid scintilation counter (ALOKA Liquid Scintilation system. SC-903), 0.5 ml NCS tissue solution and 6 ml scintilation fluid were added to the sample. The inhibition rate

of ^{3}H-TdR and ^{3}H-UR uptake was determined by (1-cpm of sample/cpm of control) × 100. The increase rate of the early stage and the late stage was calculated by (cpm of sample at 10 days/cpm of sample at 2 days ÷ cpm of control at 10 days/cpm of control at 2 days) × 100 and (cpm of sample at 15 days/cpm of sample at 7 days ÷ cpm of control at 15 days/ cpm of control at 7 days) × 100, respectively.

RESULTS

1. Growth curve

CDDP 0.1 μg/ml resulted in cell growth kinetics similar to those of the control. With CDDP 1.0 μg/ml the cells tended to show inhibition of proliferation up to 7 days and then to reveal regrowth. The concentration of 10 μg/ml resulted in reduction of number of cells, but not marked reduction, so that a constant number was seen after 7 days and viable cells of 1.1 × 10^4 cells/ml remained at 10-15 days (Fig. 1).

2. Colony forming assay

Table 1 shows the number of colonies of PC-1 cells at days 7 and 15. The number of colonies of the control at day 7 was 39.1, however, the number at day 15 was not counted due to fusion among colonies. The inhibition rate of colony
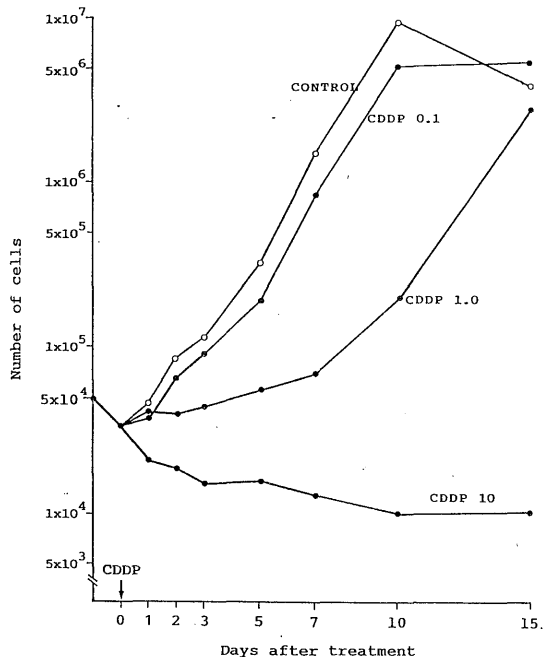

Fig. 1. Growth curve of PCl cells after treatment with several concentration of CDDP. Point, average results of 3 determination.

formation with CDDP 0.1 μg/ml at day 7 was 56.7%, whereas that at day 15 could not be evaluated due to the similar fusion. CDDP 1.0 μgml resulted in a remarkable inhibition rate of colony formation by 84.7% at day 7.

Table. 1. Evaluation of colony forming assay of PCl cells after treatment with several concentration of CDDP[a].

Concentration (μg/ml)	No. of colonies at 7 days				No. of colonies at 15 days		
	No. of cells in colonies			Total colonies	No. of cells in colonies		Total colonies
	8~15	16~31	32~		16~63	64~	
Control	20.7±14.2	9.7±3.1	8.7±3.2	39.1	Incalculability[b]		Incalculability
0.1	9.0± 1.7 (56.5)[c]	5.0±0.0 (48.5)	3.0±1.7 (65.2)	17.0 (56.5)	Incalculability		Incalculability
1.0	3.0± 1.0 (85.5)	2.0±1.7 (79.4)	1.0±0.0 (88.1)	6.0 (84.7)	1.7±1.2	6.0±2.0	7.7
10	0 (100)	0 (100)	0 (100)	0 (100)	0	0	0

a) Data are shown as the mean ± the standard deviation (n=3).
b) Numbers of colonies could not be counted as fusion of the neighbour colonies.
c) Numbers in parenthesis are percentages of inhiiition rate of colony formation.

$$\text{Inhibition rate (\%)} = (1 - \frac{\text{No. of colonies of sample cells}}{\text{No. of colonies of control cells}}) \times 100.$$

However, results in the investigation of the number of cell-forming colonies which showed 6 colonies with more than 64 cells at day 15, indicated that colonies with more than 8 cells at day 7 were favorably, repeatedly divided. Colony formation with CDDP 10 μg/ml was not recognized at days 7 and 15.

3. ^{3}H-TdR and ^{3}H-UR incorporation assay

Fig. 2 shows the change of intracellular uptake of ^{3}H-TdR and ^{3}H-UR by CDDP under indentical condition of experiment of colony formation. Cells treated with 0.1 μg/ml or 1.0 μg/ml tended to increase uptake of ^{3}H-TdR and ^{3}H-UR similar to that in the control. On the other hand, cells with CDDP 10 μg/ml were not recognized to increase uptake of ^{3}H-TdR and ^{3}H-UR up to day 7 but subsequently
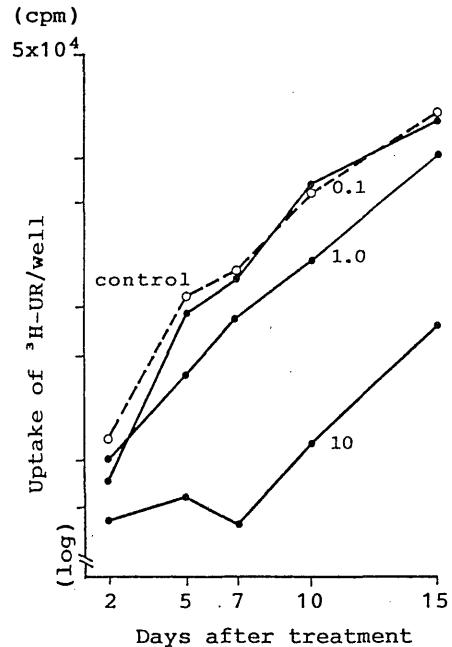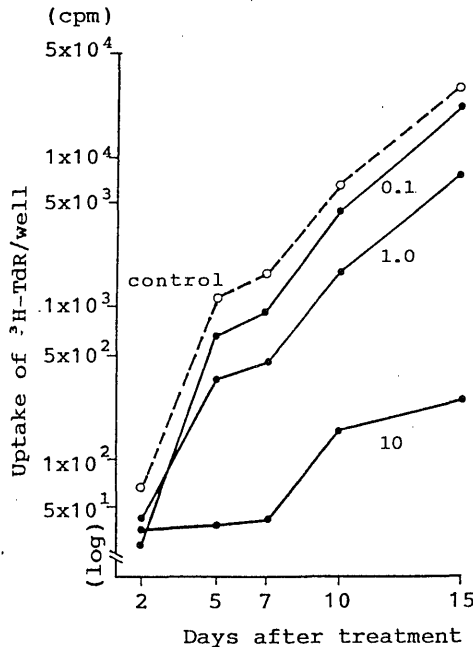

Fig. 2. Follow-up changes of ^{3}H-Thymidine and ^{3}H-Uridine uptake in PCl cells after treatment with several concentration of CDDP. Point, average results of 3 determination.

Table 2. Estimation of DNA and RNA activities by ^{3}H-Thymidine and ^{3}H-Uridine incorporation assay of PCl cells after treatment with several concentration of CDDP[a].

Concentration (μg/ml)	% Inhibition [b]				% Increase [c]			
	7 days		15 days		10 days/2 days		15 days/7 days	
	^{3}H-TdR	^{3}H-UR	^{3}H-TdR	^{3}H-UR	^{3}H-TdR	^{3}H-UR	^{3}H-TdR	^{3}H-UR
0.1	47.3	12.6	22.9	5.6	188.1	241.9	146.6	108.7
1.0	74.9	56.8	75.9	46.4	36.1	50.6	96.0	124.0
10	97.6	98.1	99.1	96.3	4.0	8.6	35.6	196.2

a) Numbers are percentages of average results (n= 3).

b) % Ithibition= $(1 - \dfrac{\text{cpm of sample cells}}{\text{cpm of control cells}}) \times 100$.

c) % Increase of 10 days/2 days= $\dfrac{\text{cpm of sample cells at 10 days}}{\text{cpm of sample cells at 2 days}} \div \dfrac{\text{cpm of comtrol cells at 10 days}}{\text{cpm of comtrol cells at 2 days}} \times 100$.

   % Increase of 15 days/7 days= $\dfrac{\text{cpm of sample cells at 15 days}}{\text{cpm of sample cells at 7 days}} \div \dfrac{\text{cpm of comtrol cells at 15 days}}{\text{cpm of comtrol cells at 7 days}} \times 100$.

tended to increase. This change was investigated by means of comparisons of inhibition rate (% inhibition) and increase rate (% increase) (Table 2). Inhibition rate of ^{3}H-TdR with 0.1 μg/ml or 1.0 μg/ml at day 7 was comparable with inhibition rate of colony formation at day 7 and higher than inhibition rate of ^{3}H-UR. Cells treated with CDDP 10 μg/ml which did not show colony formation revealed a remarkable inhibition rate of ^{3}H-TdR and ^{3}H-UR. Although reduction of inhibition rate of ^{3}H-TdR and ^{3}H-UR was seen with CDDP 0.1 μg/ml at day 15, a similar inhibition rate was observed for cells with 1.0 μg/ml and with 10 μg/ml at day 7 and 15. On the other hand, an increased rate of ^{3}H-TdR and ^{3}H-UR from day 2 to 10 resulted in a higher rate with lower concentration of CDDP. Increase rate with 0.1 μg/ml from day 7 to 15 decreased in both ^{3}H-TdR and ^{3}H-UR more than those from day 2 to 10, especially, the rate for ^{3}H-UR. On the other hand, the rate from day 7 to 15 with 1.0 or 10 μg/ml was higher than that from day 2 to 10 for ^{3}H-TdR and ^{3}H-UR, and the rate for ^{3}H-UR was higher than that for ^{3}H-TdR. Especially, the rate for ^{3}H-UR with 10 μg/ml from day 7 to 15 was the highest in the later stage. That is, RNA synthesis tended to become active with CDDP of high concentration at the later stage.

4. *^{3}H-TdR and ^{3}H-UR incorporation assay using other drugs*

A comparison study was conducted between variation of uptake of ^{3}H-TdR and ^{3}H-UR with CDDP 10 μg/ml and variation of uptake with MMC 5.0 μg/ml or ADM 1.0 μg/ml which reveals similar cell growth kinetics to CDDP 10 μg/ml (Fig. 3). Variation of uptake of ^{3}H-TdR with MMC or ADM tends to be similar to that with CDDP, although it was a low value. However, variation of uptake of ^{3}H-UR with MMC or ADM after 7 days did not result in an increase of uptake, which was different to results of CDDP.

DISCUSSION

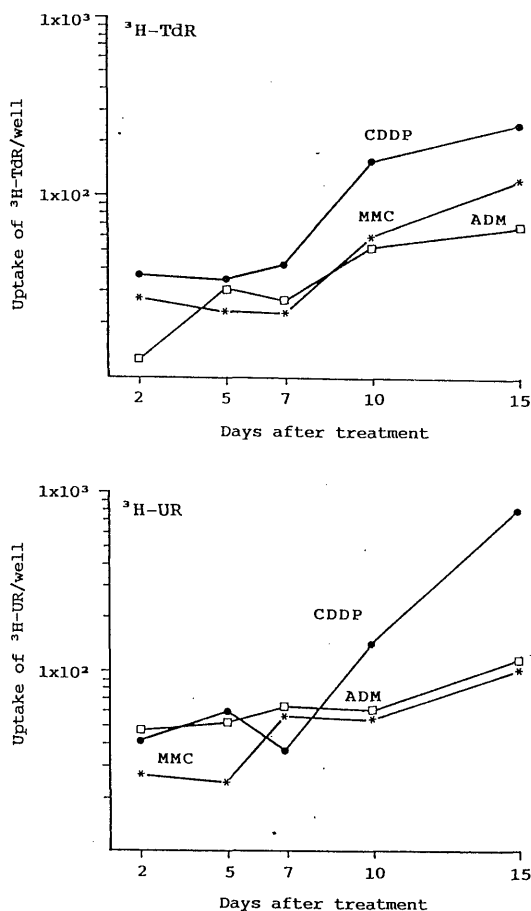CDDP is considered to be a DNA synthesis inhibitor which shows antitumor effect by

Fig. 3. Follow-up changes of ^{3}H-Thymidine and ^{3}H-Uridine uptake in PCl cells after treatment with CDDP 10 μg/ml, ADM 1.0 μg/ml and MMC 5.0 μg/ml. Point, average results of 3 determination.

means of interstrand cross-links or intrastrand cross-links with DNA[6),12)]. HowLE et al.[13)] also reported that although long reduction of DNA synthesis recognized using ^{3}H incorporation assay, reduction of RNA synthesis and protein was seen transiently, and inhibition of DNA synthesis consequently played an important rule for antitumor effect. Inhibition rate measured by ^{3}H incorporation assay in this experiment corresponded to results of HowLE et al. However, it is necessary to think about the problem of recovery from damage when antitumor effect is considered. Damaged cells with 1.0 μg/ml of CDDP which is known for the presence of the repair mechanism demons-

trated regrowth and reversibility in this study. Repair with CDDP, which has been known due to action of DNA excision repair mechanism, has been reported to be related to the residual amount of platinum in DNA[5]. In addition, recent reports indicated that SOS response is induced by many DNA damaging agents, and mutagenesis and cytotoxicity by CDDP is affected by the SOS repair mechanism with sulA fusion mutant induction[7]. It is generally known that induction of SOS response occurs by activation of RectA protein by DNA damage and subsequent stimulation of cleavage of LexA protein. During this response, it is supposed that intracellular metabolism associated with repair may be activated[14]. However, the most basic long-term experiment with CDDP in nucleic acid metabolism seems not to have been conducted. Thus, variation of early metabolism (days 2-10) and later metabolism (days 7-15) was evaluated as increase rate (follow-up variation) of ^{3}H-TdR and ^{3}H-UR uptake in treated cells against that in non-treated cells. In the early stage, increase rate of ^{3}H-UR was higher than that of ^{3}H-TdR at any concentration of CDDP, and a higher rate of ^{3}H-UR was recognized at lower concentration of CDDP. In contrast, in the later stage, ^{3}H-UR showed a higher rate at higher concentration of CDDP, and the highest rate was seen at 10 μg/ml. These results indicated that RNA metabolism, rather than DNA metabolism, becomes active first and becomes more active at higher concentration of CDDP in recovery of damaged cells. It was not determined if such a tendency is a specific phenomenon for CDDP. Also, the tendency in MMC and ADM which have not been generally known for presence of the repair mechanism was not clarified.

Studies of CDDP with respect to presence of repair mechanism have been progressing ; CDDP-damaged cells is known to be reversible. This study on the nucleic acid metabolism proved that RNA metabolism is activated first in the repair process of CDDP-damaged cells. Especially, it is remarkable that activity of RNA metabolism was observed in 10 μg/ml CDDP which did not have the capacity for colony formation. In additon, BLM which is

used in combination of CDDP is known to have the repair mechanism[8]-[10]. NISHIKORI et al.[8] reported that Actinomycin D of RNA synthesis inhibitor inhibited the action of recovery from BLM damage, because it was considered that Actinomycin D possibly inhibited RNA metabolism in the repair process. If intracellular activity of RNA metabolism is necessary for recovery and reproliferation of anticancer-induced damage cells, it is emphasized that RNA synthesis inhibitors may be one of beneficial drugs administerd after induction therapy of CDDP and/or BLM.

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