Sequence and Time Interval in Combination of Irradiation and Cis-Diamminedichloroplatinum in C3H Mouse Fibrosarcoma

— Tumor Size Effect —

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Experiments have been carried out with C3H mouse fibrosarcoma (FSa II) to determine the effect of different sequence and time intervals between irradiation and administration of cis-diamminedichloroplatinum (cis-DDP) with gross tumors (6 mm in diameter), microscopic tumors (3 days after transplantation of 10^3 cells) and cells in culture. The drug was administered either 24, 12, 8, 4, 2, 1, 0.5 hour before irradiation, immediately before irradiation, or 0.5, 1, 2, 4, 8, 12, 24 hours after irradiation. In case of *in vivo* studies, tumor growth delay was used as an end point. Clonogenic cell surviving fraction was used for *in vitro* studies.

Tumor growth delay for gross tumor after 10 Gy radiation plus 10 mg/kg cis-DDP ranged from 6.3 to 10.66 days and the enhancement ratio ranged from 1.37 to 2.23. The most effective combination was when cis-DDP was given 4 hours before irradiation. Tumor growth delay for microscopic tumor after 5 Gy of radiation and 5 mg/kg of cis-DDP ranged from 3.55 to 11.98 days with enhancement ratio from 2.05 to 6.92. Microscopic tumors showed response significantly greater than additive in every time interval and the most effective treatments were when cis-DDP was given 2 and 1 hour before irradiation. In *in vitro* experiment, the surviving fraction after 6 Gy of radiation and 1 hour exposure to 4 μM cis-DDP fluctuated as a function of time between treatments, but the difference between maximum and minimum surviving fractions was very small.

According to the above results the sequence and time interval between irradiation and chemotherapy is very critical especially for the management of microscopic tumors as in the case of postoperative adjuvant treatment.

Key Words: Irradiation, Cis-diamminedichloroplatinum, Sequence and time interval

INTRODUCTION

In recent years there has been increasing clinical use of combined modality therapy in which patients have received irradiation and cytotoxic drug treatments either concurrently or sequentially. However, interactions of a cancer chemotherapeutic drug and radiation are complex and extremely difficult to evaluate clinically and experimentally. There are a number of conceptually different ways in which chemotherapy can improve the results of radiotherapy[1]. The addition of chemotherapy to radiation regimens has been directed toward both (a) increased local control and (b) adjunctive therapy of micrometastases present at the time of treatment of the primary site[2].

Because of the nature of the kinds of subcellular damage caused by ionizing radiation and cytotoxic drugs, it is reasonable to expect that direct interactions may occur and that time sequence may be an important factor in the response to the combined modality therapy. In most clinical protocols, however, little attention has been given to the optimal time interval between irradiation and drug administration. Especially for postoperative residual microscopic tumors, the investigation to find out the best time interval between chemotherapy and radiation has not been done yet. Tumor cell kinetics and tumor blood supply of these microscopic tumors may be quite different from those of gross tumors.

Experiments have been carried out with C3H mouse fibrosarcoma to determine the effect of different sequence and time intervals between irradiation and administration of cis-DDP in gross tumors, microscopic tumors, and cells in culture.

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MATERIALS AND METHODS

1. *In Vivo* Experiment

1) Experimental Animal and Tumor System

8 to 14 week old C3Hf/Sed mice weighing 20-30 g were used for this study. These mice were obtained from Edwin L Steel Laboratory of Massachusetts General Hospital and were bred and maintained in defined flora mouse colony in Radiation Biology Laboratory of Cancer Research Center of Seoul National University. These mice have only 4 kinds of Clostridium (C. 356, C. inoculum, C. bareki, C. clostridiformis) and have no viruses and no bacteria[3,4]. Fifth generation isotransplants of the spontaneous fibrosarcoma, FSa II (poorly differentiated fibrosarcoma) were used throughout these experiments. FSa II is very weakly immunogenic. Tumor material for inoculation was obtained by sterile dissection of flank tumors. Macroscopically viable tumor tissue was minced into fine pieces and single cell suspensions were prepared by trypsinization. Viable tumor cell number was counted based on trypan blue exclusion method and diluted appropriately for adjustment of cell count. One thousand viable tumor cells mixed with lethally irradiated (120 Gy *in vitro*) 2×10^5 tumor cells were transplanted into right leg muscle in an inoculum volume of 5 μl.

2) Irradiation and Drug Exposure

Experiments were carried out with the drug given 24, 12, 8, 4, 2, 1, 0.5 hour before irradiation, immediately before irradiation, or 0.5, 1, 2, 4, 8, 12, 24 hour after irradiation in gross tumor (6 mm in diameter) groups and microscopic tumor (3 days after transplantation of 10^3 cells) groups. Ten mice were used in each group. All irradiations were performed on unanesthesized mice using Cs-137 irradiator, which provides parallel opposed 3×3 cm fields. The dose rate was about 536 cGy per minute during these experiments. Each mouse was restrained within an individual box (Fig. 1) specially designed for immobilization and protection from contamination. During irradiation clean air through air filter was provided to mice in box by air compressor. Single radiation dose of 10 Gy was given for gross tumors and 5 Gy for microscopic tumors. Cis-DDP was dissolved in sterile saline and injected intraperitoneally in a volume of 0.01 ml/g. On the basis of pilot study data a dose of 10 mg/kg for gross tumors and 5 mg/kg for microscopic tumors of cis-DDP was given.

3) Tumor Growth Delay

After treatment the 3 perpendicular tumor diameters were measured daily with Vernier caliper. Tumor volumes were calculated from measurements of length, width, and height on the assumption that the tumors were hemi-ellipsoids.

$V=4/3\pi\times R_1/2\times R_2/2\times R_3/2$

Individual tumor growth curve was plotted for each of the tumors and tumor growth time to reach 500 mm^3 was estimated. Tumor growth delay was used as an end point and this was the difference of tumor growth time of experimental and control mice to reach 500 mm^3 after treatment. The additive band was obtained by adding the growth delays for the radiation and drug controls. Enhancement ratio

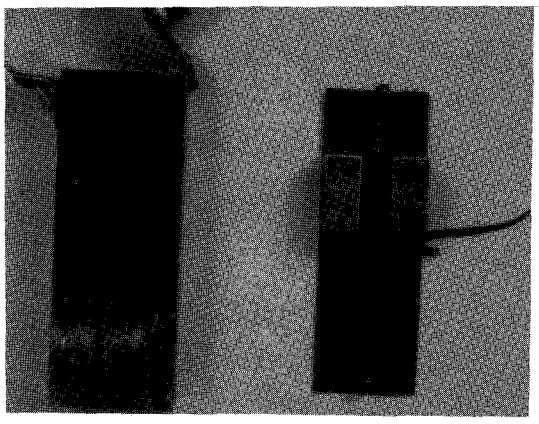

Fig. 1. Aluminum plate and box specially designed for local irradiation of FSa II bearing mouse leg without anesthesia.

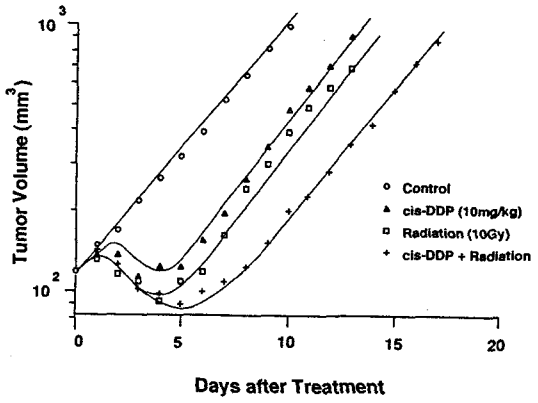

Fig. 2. Tumor growth curves of 6 mm FSa II tumors after the various treatments.

Table 1. Tumor Growth Delay and Enhancement Ratio by Cis-DDP for Gross (6 mm) FSa II Tumors

Groups	Tumor growth delay ±1 S.D. (days)	Enhancement ratio
R	4.61±1.64	
C	3.80±1.33	
C-24-R	6.30±0.34*	1.37
C-12-R	7.89±0.20	1.71
C- 8-R	8.01±0.40	1.74
C- 4-R	10.66±0.69*	2.31
C- 2-R	8.18±0.84	1.77
C- 1-R	9.04±0.52	1.96
C-0.5-R	9.83±0.68	2.13
C- 0-R	10.27±0.28*	2.23
R-0.5-C	7.86±0.57	1.70
R- 1-C	8.35±0.51	1.81
R- 2-C	9.35±0.54	2.03
R- 4-C	8.81±0.67	1.91
R- 8-C	8.39±0.19	1.82
R-12-C	8.35±0.46	1.81
R-24-C	7.12±1.03	1.54

Note: R: irradiation (10 Gy) C: cis-DDP (10 mg/kg)
C-24-R: irradiation 24 hours after cis-DDP
*: Tumor growth delay is significantly different from additive line. ($p<.05$)

was calculated from following equation.

$$\text{Enhancement ratio} = \frac{\text{Tumor growth delay with cis-DDP}}{\text{Tumor growth delay without cis-DDP}}$$

2. *In Vitro* Experiment

Single cell suspension of FSa II cells was prepared and appropriate number of cells were plated in T-25 flask at appropriate concentrations to produce 50 to 100 colonies per flask. Before treatment controls were checked for plating efficiency and multiplicity. The same time intervals of cis-DDP and irradiation were chosen for the *in vitro* assay experiments for comparison with the *in vivo* experiment. 6 Gy of radiations was delivered using Cs-irradiator. Cis-DDP was diluted appropriately in isotonic saline to allow spiking of the cell culture medium (5 ml per flask) with 4 μM of drug solution. Cells were exposed to cis-DDP for one hour. For simultaneous treatment radiations was given 30 min after starting the drug incubation. 10-14 days after 37℃ incubation clonogenic survival was determined by counting colonies of 50 and more cells.

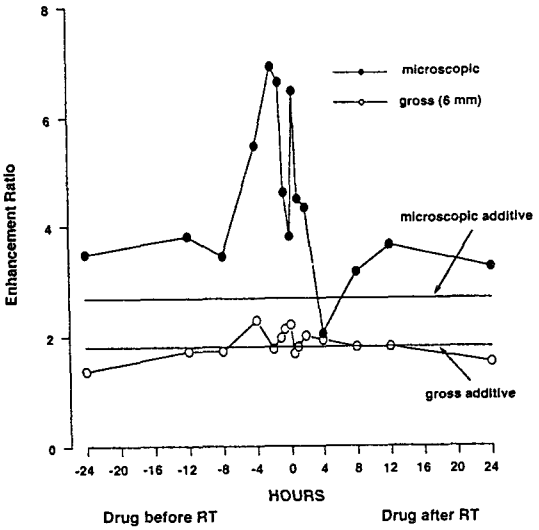

Fig. 3. Enhancement ratio according to the sequence and time interval from irradiation to cis-DDP in gross (6 mm in diameter) and microscopic (3 days after 10^3 cell transplantation) tumors of FSa II. Enhancement ratios are much higher in case of microscopic tumor even with lower dose of cis-DDP (5 mg/kg) compared to those with gross tumor (10 mg/kg). Each point represents data from 10 mice.

RESULTS

Average growth curves for gross tumors treated with 10 Gy of irradiation alone, 10 mg/kg of cis-DDP alone, and combinations of these 2 agents are given in Fig. 2. Radiation or cis-DDP induced tumor growth delay without change of slope. For gross tumor, tumor growth time to reach the tumor volume of 500 mm^3 for control group was 6.69±1.19 days (Table 1). Tumor growth delay by 10 Gy of irradiation and 10 mg/kg of cis-DDP was 4.61 and 3.80 days, respectively. The additive band obtained by adding the growth delays for the radiation and drug controls was 8.41 days. Tumor growth delay for gross tumor after irradiation plus cis-DDP ranged from 6.3 to 10.66 days and the radiation enhancement ratio ranged from 1.37 to 2.23 (Fig. 3). The most effective combination was cis-DDP followed by radiation with 4, 0.5 and 0 hour interval. Radiation followed by cis-DDP with 2 hour interval was also very effective.

For microscopic tumors, tumor growth time to

Table 2. Tumor Growth Delay and Enhancement Ratio by Cis-DDP for Microscopic (3 day old 1,000 celis) FSa II Tumors

Groups	Tumor growth delay ±1 S.D. (days)	Enhancement ratio
R	1.73±0.53	
C	2.94±0.63	
C-24-R	6.04±0.48	3.49
C-12-R	6.58±0.90	3.80
C- 8-R	5.96±0.73	3.45
C- 4-R	9.46±0.76*	5.47
C- 2-R	11.98±1.14*	6.92
C- 1-R	11.49±1.62*	6.64
C-0.5-R	7.98±0.93*	4.61
C- 0-R	6.59±1.09	3.81
R-0.5-C	11.18±1.29*	6.46
R- 1-C	7.76±0.60*	4.49
R- 2-C	7.54±0.81*	4.36
R- 4-C	3.55±0.52	2.05
R- 8-C	5.46±1.51	3.16
R-12-C	6.31±1.46	3.65
R-24-C	5.64±0.54	3.26

Note: R: irradiation (5 Gy) C: cis-DDP (5 mg/kg)
C-24-R: irradiation 24 hours after cis-DDP
*: Tumor growth delay is significantly different from additive line. ($p<.05$)

reach the tumor volume of 500 mm^3 for control group was 17.44±0.31 days (Table 2). Tumor growth delay by 5 Gy of radiation and 5 mg/kg of cis-DDP was 1.73 and 2.94 days, respectively. The additive band was 4.67 days. Tumor growth delay for microscopic tumors after irradiation plus cis-DDP ranged from 3.55 to 11.98 days with enhancement ratio between 2.05 and 6.2 (Fig. 3). Cis-DDP followed by radiation with 1 to 2 hour interval and radiation followed by cis-DDP with 0.5 hour interval were most effective.

In contrast with *in vivo* experiment, sequence and time interval showed minimal effect in *in vitro* study. Tumor cell survival after 6 Gy of irradiation and I hour exposure to 4 μM of cis-DDP ranged from 0.0009 to 0.0020 (Table 3) with enhancement ratio from 1.32 to 2.64 (Fig. 4).

DISCUSSION

The ability of cis-DDP to enhance radiation

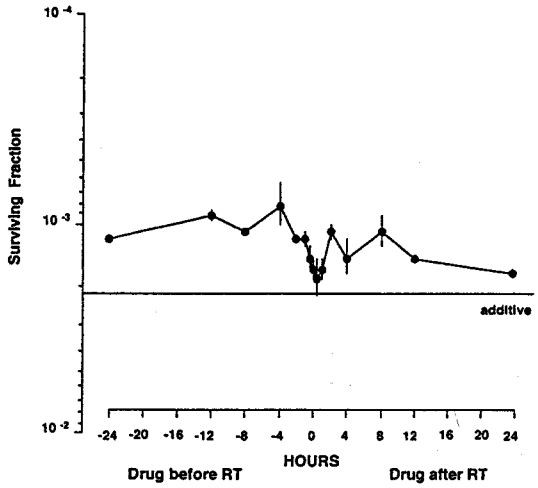

Fig. 4. Cell survival fraction extimated *in vitro* after 6 Gy of radiation and 1 hour exposure to 4 μM of cis-DDP. Combination effect is superior to simple addition of the two, but no significant difference along the sequence and time interval is demonstrated.

Table 3. Cell surviving Fraction after Irradiation and Cis-DDP Treatment for FSa II Tumor Cells in Culture

Groups	Surviving fraction±1 S.D.
R	0.0350±0.00200
C	0.0680±0.00200
C-24-R	0.0013±0.00006*
C-12-R	0.0010±0.00006*
C- 8-R	0.0012±0.00001*
C- 4-R	0.0009±0.00020*
C- 2-R	0.0013±0.00005*
C- 1-R	0.0013±0.00010*
C-0.5-R	0.0016±0.00020*
C- 0-R	0.0018±0.00020*
R-0.5-C	0.0020±0.00040
R- 1-C	0.0018±0.00020*
R- 2-C	0.0012±0.00010*
R- 4-C	0.0016±0.00030*
R- 8-C	0.0012±0.00020*
R-12-C	0.0016±0.00005*
R-24-C	0.0019±0.00010*

Note: R: irradiation (6 Gy) C: cis-DDP (4 μM)
C-24-R: irradiation 24 hours after cis-DDP
*: Cell surviving fraction is significantly different from additive line. ($p<.05$)

effects has been observed in a large number of *in vitro* studies[5], and several mechanisms have been advanced to explain this. First, it may act as a hypoxic cell sensitizer. Hypoxic cells do not exist in microscopic tumors and cells in culture so we can not expect the hypoxic cell sensitizing effect in these experiment. Second, the drug may inhibit the repair of sublethal or potentially lethal damage. Third, the drug may induce chromosomal aberrations or the formation of DNA inter and especially intrastrand crosslinks. Fourth, it may act as a depletor of or binder to cellular repair proteins[6]. These mechanisms provide neither a full nor an adequate explanation for the kind of radiation sensitization produced by cis-DDP in the present tumor experiments. More research on the interaction of cis-DDP and radiation is needed to understand the mechanism involved and to improve the radiosensitizing effects of this drug.

Begg et al[7] reported that potentiation between chemotherapy and radiation is markedly dose dependent. 50 mg/kg cytoxan plus 800 cGy showed no potentiation, 120 mg/kg cytoxan plus 800 cGy showed significant but variable potentiation whereas 150 mg/kg plus 1500 cGy showed consistent potentiation. The cis-DDP dose dependence for the effect of combinations of this durg with radiation emphasizes the nedd to establish the maximum effective cis-DDP dose in experiments.

In our experiments, the most effective treatments for gross tumor were when cis-DDP was given 4 hour before irradiation. For microscopic tumor the most effective combination was cis-DDP followed by radiation with 0.5 and 0 hour interval. For cis-DDP, Overagaard and Khan[8] observed a marked enhancement of the radiation response at administration 30 min before irradiation. But the main feature of several data is the lack of evidence for a consistent trend towards maximum tumor response at any specific interval. These experimental data support that there may be variations according to both tumor types and chemotherapeutic agents.

Microscopic tumor showed combined response significantly greater than additive and the most effective time of combination is shorter than for gross tumors. These difference may come from that microscopic tumors have 1) shorter cell cycle time[9], 2) larger growth fraction[10], 3) higher drug concentration in tumor, and 4) fasten drug delivery because of abundance of blood supply[11].

These results suggest that the sequence and time interval is very critical in combination treatment of radiation and cis-DDP especially in case of microscopic tumors as in the case of adjuvant treatment.

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= 국문초록 =

C3H 마우스 섬유육종에 있어서 방사선 조사와 Cis-diamminedichloroplatinum의 병용시 순서 및 시간간격의 영향

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방사선 조사와 cis-diamminedichloroplatinum(이하 cis-DDP라 함)의 병용시 최대효과를 얻기 위한 병용순서와 시간간격을 알아보기 위하여 C3Hf/Sed 마우스의 하지근육에 섬유육종(fibrosarcoma: FSa II)을 이식하여 발생시킨 육안적 종양(직경 6 mm)과 미시적 종양(1,000개의 종양세포 이식후 3일된 종양)을 대상으로 실험하였고 동시에 생체외 실험을 병행하였다.

육안적 종양은 10 mg/kg의 cis-DDP를 10 Gy의 방사선 조사전 24, 12, 8, 4, 2, 1시간 및 30분과 동시 그리고 방사선 조사후 30분 1, 2, 4, 8, 12, 24시간에 복강내에 투여하였고 미시적 종양은 같은 시간간격으로 5 mg/kg의 cis-DDP를 5 Gy의 방사선과 병용하여 투여하였다. 실험의 결과는 종양을 매일 3차원적으로 측정하여 성장곡선을 그린 뒤 성장 지연기간을 계산하여 판정하였다. 동시에 생체외 실험은 같은 시간간격으로 4 μM의 cis-DDP 1시간 노출과 6 Gy의 방사선을 조사한 뒤 실험후 10내지 14일에 세포집락의 수를 측정하여 세포 생존분획을 얻었다.

육안적 종양에 대한 결과는 방사선과 항암제를 병용함으로써 방사선 조사전 4시간부터 방사선 조사후 4시간까지는 부가선(additive line) 이상의 효과를 보였으나 병용시간 간격이 4시간 이상인 군에서는 부가선 이하의 효과를 보였다. 가장 효과적인 병용시간은 cis-DDP를 방사선 조사전 4시간에 투여한 군으로 성장지연은 10.66일이었다. 미시적 종양에서는 방사선과 항암제를 병용함으로써 방사선 조사후 4시간에 cis-DDP를 투여한 군을 제외하고는 모든 군에서 부가선 이상의 효과를 얻었으며 그 중에서도 cis-DDP를 방사선 조사전 2시간, 1시간에 투여한 군에서 가장 효과가 높았다. 생체외실험에서는 병용의 효과는 모든 시간간격에서 부가효과(additive effect) 이상이었으나 시간간격에 따른 차이는 적었다.

이상의 결과에서 항암제와 방사선을 병용하여 치료할 경우 특히 미시적종양에서는 병용순서 및 시간간격에 따라 암치료 효과에 미치는 영향이 지대하므로 병용치료시 적절한 순서 및 시간간격의 선택이 필수적임을 알 수 있었다.

중심단어 : 방사선 조사, Cis-diamminedichloroplatinum, 병용순서와 시간간격