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ORIGINAL ARTICLE

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Detection and cytotoxicity of cisplatin-induced superoxide anion in monolayer cultures of a human ovarian cancer cell line

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Abstract Superoxide anions (O_2^-) generated by cisplatin [cis-diamminedichloroplatinum (II), DDP] were determined by measuring the chemiluminescence from the luminescence probe, 2-methyl-6-[*p*-methoxyphenyl]-3,7-dihydroimidazo[1,2-*a*]pyrazin-3-one (methyl *Cypridina* luciferin analog, MCLA), in monolayer cultures of a human ovarian cancer cell line (A2780) in physiological saline at pH 7.0. In a time-course study, chemiluminescence of MCLA (C-MCLA) showed a peak level at 10 min and a background level at 60 min after the addition of DDP. The intensity of C-MCLA increased with increasing concentrations of DDP or MCLA in a limited concentration range, and was significantly correlated ($r = 0.960$) with the number of A2780 cells. DDP-induced C-MCLA was completely inhibited by the addition of the O_2^- scavenger, superoxide dismutase (SOD). However, SOD did not decrease DDP cytotoxicity in terms of clonogenic cell survival. These findings suggest that DDP generates extracellular O_2^-, probably by interaction with the cellular membrane in A2780 cells, and O_2^- does not lead to cellular damage.

Key words Cisplatin · Superoxide anion · Methyl *Cypridina* luciferin analog (MCLA) · Chemiluminescence · Human ovarian cancer cell line

Introduction

Cisplatin [cis-diamminedichloroplatinum (II), DDP] is an effective anticancer agent in the treatment of a wide range of human cancers [4]. It has been proposed that reactive oxygen species (ROS) play a crucial role in DDP-induced cytotoxicity [7, 9, 21], although the major mechanism of DDP cytotoxicity is the inhibition of DNA synthesis through inter- and intrastrand cross-linking [23]. Several studies have demonstrated that DDP induces ROS in murine macrophage monolayers [13, 15, 16] and human polymorphonuclear leukocytes [1]. However, in these studies the primary function of these phagocytes, e.g. the release of O_2^- and H_2O_2, was investigated.

The present study was performed to evaluate the involvement of ROS in DDP cytotoxicity in cancer cells. We have previously reported that the interaction of DDP with purified cellular DNA generates O_2^- in cell-free systems [5, 6]. In the course of our efforts to detect ROS released from living cells as a result of DDP treatment, we found a simple method of detecting DDP-induced O_2^- in a human ovarian cancer cell line, cultured as a monolayer, by measuring the chemiluminescence from the luminescence probe, 2-methyl-6-[*p*-methoxyphenyl]-3,7-dihydroimidazo[1,2-*a*]pyrazin-3-one (methyl *Cypridina* luciferin analog, MCLA), and found that O_2^- induced in this way did not lead to cellular damage.

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Materials and methods

Chemicals

Eagle's minimum essential medium (MEM) and kanamycin were obtained from Nissui (Tokyo, Japan), fetal bovine serum (FBS) was from GIBCO (Grand Island, N.Y.), glutamine was from Wako (Tokyo, Japan), DDP was from Nippon Kayaku (Tokyo, Japan), MCLA was from Tokyo Kasei Kogyo (Tokyo, Japan), superoxide dismutase (SOD, EC.1.15.1.1, activity 5280 U/mg) was from Boehringer Mannheim (Mannheim, Germany), and diethyldithiocarbamic acid (DDC) was from Sigma (St. Louis, Mo.).

Cell line and culture

A2780, a DDP-sensitive ovarian cancer cell line derived from an untreated patient was provided by T.C. Hamilton (Fox Chase Cancer Center, Philadelphia, Pa.). The cells were maintained as

monolayers at 37 °C under a humidified atmosphere of 5% CO_2/95% air (CO_2 incubator) in MEM supplemented with 10% (v/v) FBS, 60 mg kanamycin/ml and 0.3 mg glutamine/ml (culture medium).

Luminescence measurement

The cells were plated at 2×10^5 cells/dish (3.5 cm diameter) in culture medium and allowed to grow almost to confluence for 4 days. They were then washed five times with physiological saline adjusted to pH 7.0 with $NaHCO_3$, and 3 ml of the solution containing the desired concentrations of MCLA (reaction mixture) was added. Preliminary experiments showed that this physiological saline produced the lowest background level of MCLA chemiluminescence (C-MCLA) of the buffers tested including phosphate-buffered saline, Hank's solution or culture medium, which were without adverse effect on living cells. Immediately after various amounts of DDP were added to the reaction mixture, C-MCLA was measured at 26 °C for 16 to 80 min in the absence or presence of SOD with a photon counter (model C5410) and a photomultiplier (model E1341; Hamamatsu Photonics, Tokyo, Japan) without a filter. MCLA alone was used instead of DDP for observing the background level of C-MCLA. SOD was inactivated by the method of Heikkila et al. [3]. Briefly, SOD (1 mg/ml) and DDC (10^{-2} *M*) were incubated alone and together in distilled water for 1.5 h at 37 °C. The samples were then dialyzed against 500 volumes of distilled water at 4 °C for 16 h, and again with fresh distilled water for 3 h. Aliquots were used for the C-MCLA assay. In some experiments, no cells and ethanol-fixed monolayer cells were used. The findings were expressed as the time-course of C-MCLA or counts per second (cps) of the peak level (background level subtracted) obtained from the time-course assay.

Cell viability tests

Confluent cell layers in culture medium were washed five times with physiological saline and incubated for 60 min at 26 °C without CO_2 (under the same conditions as for the C-MCLA assay) in a reaction mixture containing 6 n*M* MCLA or fresh culture medium in the presence or absence of 40 or 70 μ*M* DDP. After treatment with DDP, cells were washed twice, incubated for 5 h at 37 °C in fresh culture medium in a CO_2 incubator. The cells were then spread on the bottom of the dish, trypsinized, and counted in a Coulter counter (Coulter Electronics, Luton, UK). Cell viability was determined in terms of percentages of the control (0 μ*M* DDP in the culture medium), because the preliminary trypan blue dye exclusion tests showed that spread cells were viable.

Survival studies

Confluent cell layers in a culture dish were treated with various concentrations of DDP in physiological saline (pH 7.0) for 60 min at 26 °C without CO_2 (under the same condition as for the C-MCLA assay) in the presence or absence of SOD, and then washed three times with the solution. The cells were harvested with trypsin and were seeded into ten dishes (6 cm diameter) containing MEM with 10% FBS, at 5×10^2 cells/dish for each treatment. After incubation at 37 °C for 9 days, the cultures were rinsed with physiological saline, fixed with ethanol, stained with Giemsa and scored for survival. This was done by counting colonies of more than 50 cells under a stereoscopic microscope. A2780 cells had plating efficiencies of approximately 66%.

Statistical analysis.

For statistical analysis, Student's *t*-test was employed and a *P*-value of < 0.05 was considered significant.

Results

Measurement of C-MCLA

Figure 1A shows a photon counter tracing of C-MCLA. A2780 cells were exposed to DDP at 70 μ*M* for 80 min at 26 °C in a reaction mixture containing 6 n*M* MCLA. C-MCLA increased gradually, peaked at approximately 10 min, and then decreased slowly to the background level at 60 min, after the addition of DDP. The background level of C-MCLA, which was derived from MCLA alone in A2780 cells, was 20 ± 7 cps (Fig. 1B). Figure 1C shows that C-MCLA was not induced by 70 μ*M* DDP in the absence of cells. The ethanol-fixed cell monolayers generated almost no C-MCLA (Fig. 1D).

Effects of SOD on DDP-induced C-MCLA in 2780 cells

Figure 2A shows the 40-min time-course of C-MCLA induced by DDP in A2780 cells to clarify the effects of SOD. C-MCLA increased gradually after the addition of 70 μ*M* DDP to 3 ml reaction mixture containing 6 n*M* MCLA, and subsequently showed a slow decrease. However, it dropped rapidly as soon as 0.3 μg SOD/ml was added (Fig. 2B). When 10 μl distilled water (SOD solvent) was added instead of SOD near the peak level of C-MCLA, C-MCLA was decreased by approximately 15% (419 cps before addition, 355 cps after addition, of distilled water; Fig. 2C). A similar effect to that of distilled water on C-MCLA was obtained by DDC-inactivated SOD (18.0% decrease in C-MCLA; Fig. 2D). Thus, DDC-inactivated SOD barely affected C-MCLA as an enzyme and/or protein.

Effects of MCLA on C-MCLA

Figure 3 shows the effect of MCLA concentration on the peak level of C-MCLA in A2780 cells. C-MCLA induced at 70 μ*M* DDP increased almost linearly with concentration of MCLA, and at 6 n*M* reached a plateau that was maintained at least up to 8 n*M*. Therefore, 6 n*M* MCLA was used in subsequent C-MCLA assays.

Effects of DDP on C-MCLA

Figure 4 shows the response of C-MCLA to DDP at various concentrations in A2780 cells. C-MCLA increased in a linear fashion with concentrations of DDP up to 70 μ*M*, and then remained unchanged at concentrations up to 90 μ*M*.

Effects of cell number on C-MCLA

C-MCLA induced by 70 μ*M* DDP in different numbers of cells was examined (Fig. 5). C-MCLA increased

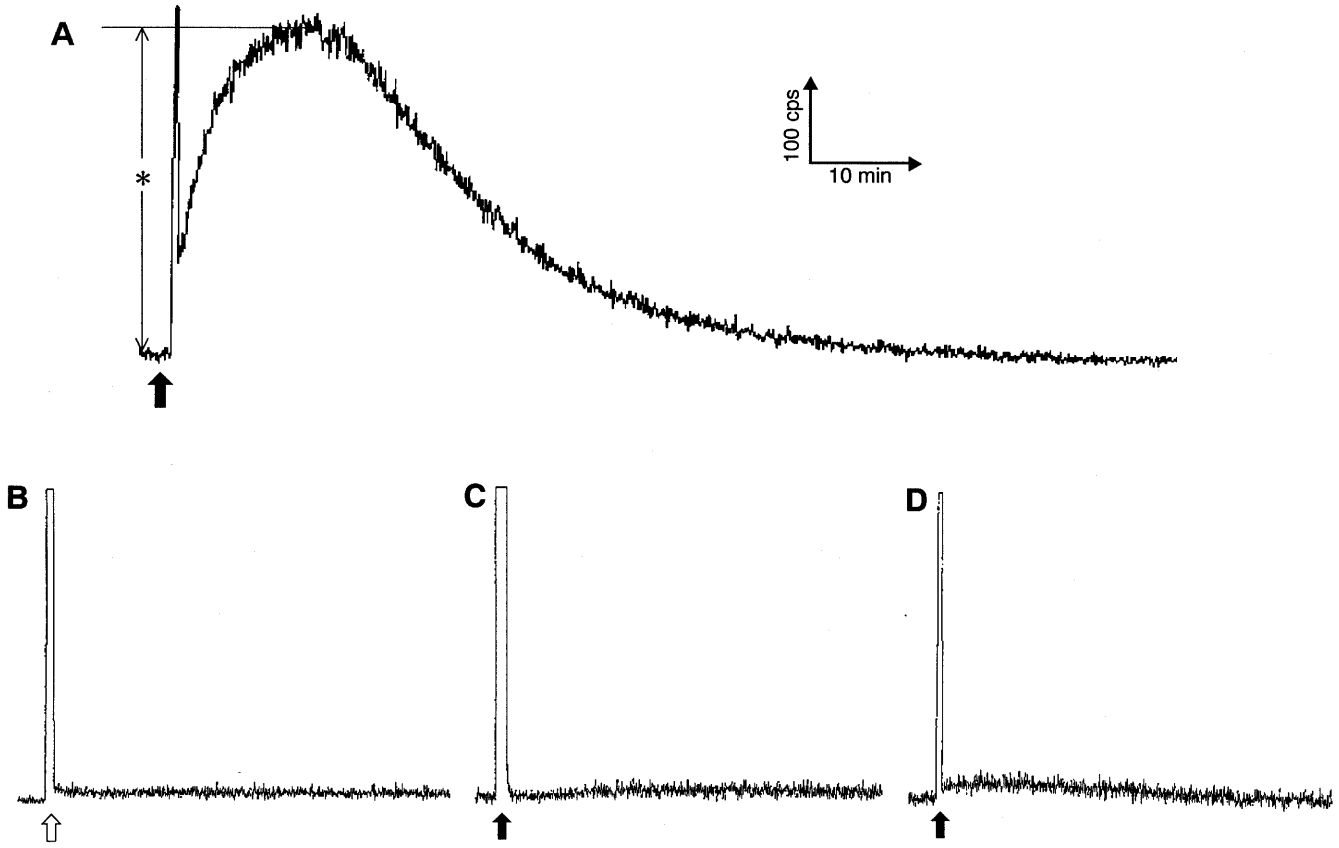

Fig. 1A–D Measurement of C-MCLA. **A** Time-course of C-MCLA. The reaction mixture (3 ml) poured over A2780 cells (4.33×10^6 cells/dish) containing 6 n*M* MCLA. The measurement of C-MCLA started immediately after the addition of 70 μ*M* DDP at 26ºC. The *asterisk* indicates the peak level of C-MCLA. **B** Control for the background level of C-MCLA without DDP. Physiological saline adjusted to pH 7.0 (3 ml) was poured over A2780 cells (4.21×10^6 cells/dish). The measurement of C-MCLA started immediately after the addition of 6 n*M* MCLA at 26ºC. **C** Control with no cells. C-MCLA was measured at 26ºC immediately after the addition of 70 μ*M* DDP to 6 n*M* MCLA in a 3-ml reaction mixture without cells. **D** C-MCLA in fixed cells. The reaction mixture (3 ml) was poured over ethanol-fixed A2780 cells (about 4.33×10^6 cells/dish) containing 6 n*M* MCLA. The measurement of C-MCLA started immediately after the addition of 70 μ*M* DDP at 26ºC. The *closed arrows* and the *open arrow* indicate the application of DDP and MCLA, respectively

with cell number from 1.20×10^5 up to 3.35×10^6 cells/dish. There was a significant correlation ($r = 0.960$, $P < 0.001$) between cell number and the level of C-MCLA.

Cell viability after C-MCLA assays

The slow decrease in C-MCLA in A2780 cells may be related to the loss of cell viability in the reaction mixture. Therefore, cell viability (survival rate) in the reaction mixture was compared with that in the culture medium with or without DDP (Table 1). Cell survival rates were 100% (control), 97.8% and 99.6% in the culture medium, 99.6%, 98.3% and 95.2% in the reaction mixture after 60 min treatment with DDP at concentrations of 0, 40 and 70 μ*M* at 26 °C, respectively. There was no significant difference between these cell survival rates. These findings indicate that the reaction mixture, even with DDP, did not affect cell viability.

Effects of SOD on DDP cytotoxicity

Figure 6 shows the survival rates of A2780 cells in the presence or absence of 0.3 μg SOD/ml after 60 min treatment at 26 °C with increasing concentrations of DDP. The survival of A2780 cells decreased with concentrations of DDP between 10 and 70 μ*M* in both the presence and absence of SOD, and treatment with DDP alone resulted in similar survival to that obtained with combinations of DDP and SOD. Thus, SOD did not affect DDP cytotoxicity at any of the DDP concentrations tested.

Discussion

MCLA has been shown to be a very sensitive and specific luminescence probe for detecting O_2^- [10, 11, 20]. It has been used to detect O_2^- generated by activated leukocytes and macrophages [10, 11, 14, 22]. We describe here the first application of the MCLA-dependent chemiluminescence method to monolayers of an ovarian cancer cell line for detecting O_2^- induced by DDP, and

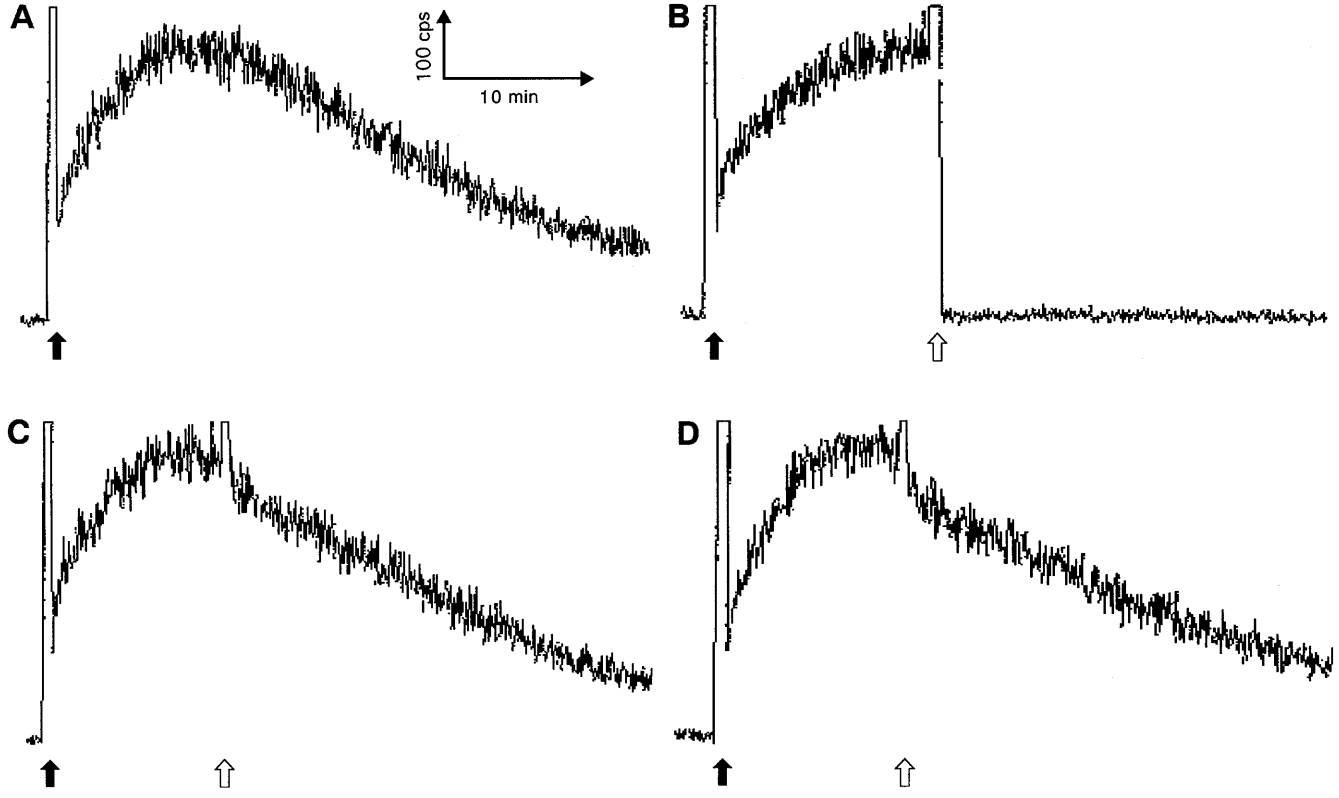

Fig. 2A–D Effects of SOD on DDP-induced C-MCLA in A2780 cells ($5.10 \pm 0.21 \times 10^6$ cells/dish). **A** C-MCLA was measured for 40 min as described in the legend for Fig. 1A. **B** SOD (10 µl, final concentration 0.3 µg/ml) was added near the peak of C-MCLA. **C** Distilled water (10 µl, SOD solvent) was added near the peak C-MCLA. **D** DDC-inactivated SOD (10 µl, final concentration 0.3 µg/ml) was added near the peak of C-MCLA. The *closed arrows* and *open arrows* indicate the application of DDP and distilled water/SOD, respectively

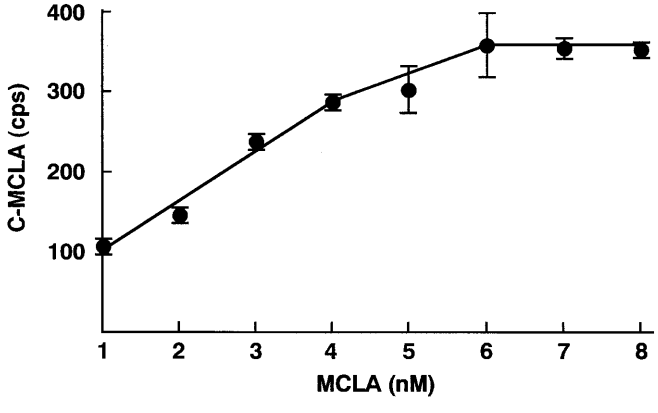

Fig. 3 Effects of MCLA concentration on DDP-induced C-MCLA. The reaction mixture (3 ml) poured over A2780 cells ($4.40 \pm 0.16 \times 10^6$ cells/dish) containing various concentrations of MCLA. The measurement of C-MCLA started immediately after the addition of 70 µ*M* DDP. The peak values of C-MCLA (asterisk in Fig. 1A) are plotted as a function of MCLA concentration. Values are counts per second (cps) of the peak level of C-MCLA (control values subtracted) (*bars* SD of triplicate determinations)

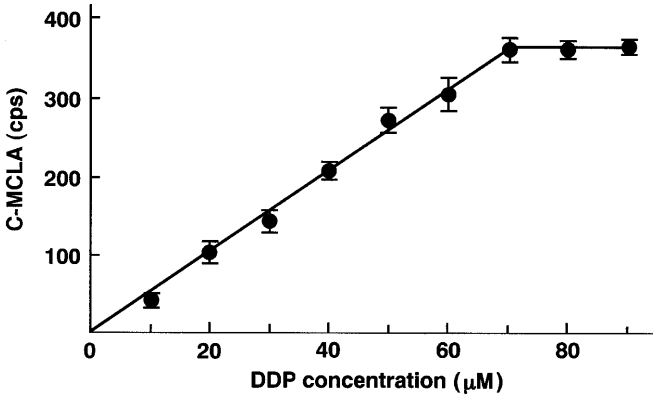

Fig. 4 Effects of DDP concentration on C-MCLA. The reaction mixture (3 ml) was poured over A2780 cells ($5.07 \pm 0.14 \times 10^6$ cells/dish) containing 6 n*M* MCLA. The measurement of C-MCLA started immediately after the addition of DDP at various concentrations. Values are counts per second (cps) of the peak level of C-MCLA (control values subtracted) (*bars* SD of triplicate determinations)

found no effect of O_2^- produced in this system on DDP cytotoxicity.

In the present study, physiological saline was used as the reaction mixture, adjusted to pH 7.0. The viability of cells was not altered after 60 min incubation at 26 °C without CO_2 in physiological saline at pH 7.0 (Table 1). Therefore, the time-dependent decrease in C-MCLA (Fig. 1A) was not due to a loss of cell viability. The level of chemiluminescence is affected by changes in pH of the medium in cell systems [2]. Oosthuizen et al. have demonstrated that MCLA produced optima closer to

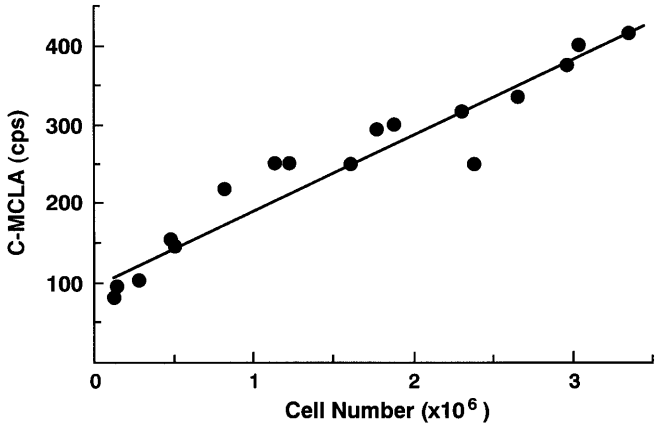

Fig. 5 Effects of cell number on DDP-induced C-MCLA. A2780 monolayer cells in culture medium were scraped off the dish bottom in different quantities with a silicon rubber, and washed five times with reaction mixture, followed by the addition of solution (3 ml) containing 6 n*M* MCLA. The number of cells in each sample was determined after C-MCLA assay. The measurement of C-MCLA was as described in the legend for Fig. 3

Table 1 Cell viability after DDP treatment in the culture medium and the reaction mixture. The initial number of cells immediately before DDP treatment was $4.62 \pm 0.26 \times 10^6$ ($100 \pm 4.8\%$). Values are percentages of the control (DDP 0 μ*M* in the culture medium) (mean ± SD from triplicate determinations)

DDP (μM)	Culture medium	Reaction mixture
0	100.0 ± 1.7[a]	99.6 ± 4.8
40	97.8 ± 5.2	98.3 ± 2.6
70	99.6 ± 1.3	95.2 ± 3.9

[a] 100% represents 4.61×10^6 cells/dish

neutral pH including pH 7.0 with a hypoxanthine/xanthine oxidase system [12]. The present system showed a similar level of DDP-induced C-MCLA at pH values in the range 6.8 to 7.2. Accordingly, the decrease in C-MCLA may be attributed to a decline in the interaction of DDP and cellular components rather than to a change in extracellular pH during the C-MCLA assay. We used confluent monolayers of cells for the C-MCLA assay to obtain high levels of C-MCLA. Confluence was not a factor. The intensity of C-MCLA depended on the number of cells per dish (Fig. 5).

DDC-inactivated SOD did not essentially affect C-MCLA, since even distilled water produced a slight decrease in C-MCLA (Fig. 2C,D). Simply mixing a reaction mixture in the course of an assay disturbed the generation of C-MCLA to a similar degree as that produced by distilled water or inactivated SOD (data not shown). Thus, it is most likely that C-MCLA disappeared through the enzymatic activity of SOD alone (Fig. 2B). Accordingly, complete suppression of C-MCLA production by the addition of the O_2^- scavenger SOD (which does not enter cells) indicates extracellular generation of O_2^- in the present system. In addition, Takahashi and Asada [19] have reported that phospholipid membranes show little permeability to O_2^-.

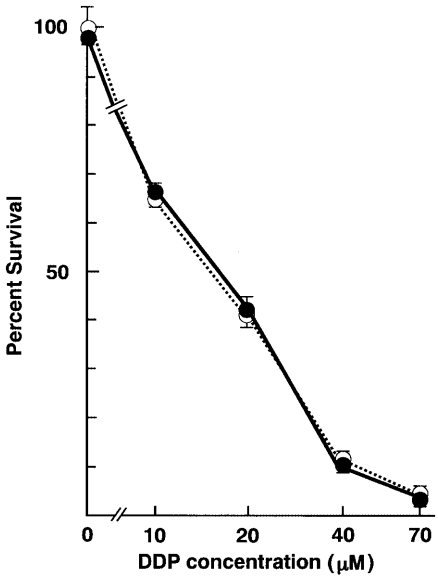

Fig. 6 A2780 cells ($5.10 \pm 0.21 \times 10^6$ cell/dish) were treated with various concentrations of DDP in physiological saline (pH 7.0) for 60 min at 26°C with (●) or without (○) 0.3 μg SOD/ml, harvested with trypsin, and assessed for clonogenic cell survival. Values are percentages of the untreated control without SOD (*bars* SD of triplicate determinations)

Hence, it is unlikely that O_2^- generated by the interaction of DDP and intracellular macromolecules, including proteins and DNA, was released extracellularly. Thus, the source of DDP-induced generation of O_2^- would be the cell membrane itself in the present system.

On the other hand, in studies of the mechanism of DDP-induced nephrotoxicity, Mcginness et al. [8] have found that orgotein (Zn-Cu-SOD) which does not enter cells ameliorates nephrotoxicity in rats. Sugihara et al. [17, 18] have found indirect evidence in rats that DDP generates free radicals that interact with membrane lipids in renal tissue and subsequently cause production of lipid peroxidation, which affects cellular structure and function. These observations suggest that DDP generates extracellular O_2^-, at least in part, in renal tissue, and this causes damage to membrane function and consequent nephrotoxicity.

However, in the present system, scavenging of O_2^- did not decrease DDP cytotoxicity (Fig. 6), indicating that O_2^- generated extracellularly by DDP did not lead to cellular damage. This inconsistency may be due to the differences between in vivo and in vitro experiments, and/or between rat kidney cells and human ovarian cancer cells. Miyajima et al. [9], by measuring the amount of intracellular dichlorofluorescence, found intracellular induction of ROS by DDP in monolayer cultures of bladder cancer cells and the involvement of ROS in DDP-induced cytotoxicity. This ROS would be generated by interaction of DDP with intracellular macromolecules. Thus, DDP may generate various ROS as a result of concomitant interactions with DNA, protein and/or membranes. Ethanol-fixed (Fig. 1D) and air-dried cells (data not shown) did not release O_2^-. This

may have been due to degeneration of target molecules for DDP in the treated cells.

In conclusion, DDP generated extracellular O_2^-, which did not take part in DDP cytotoxicity, in monolayer cultures of human ovarian cancer cells. It is suggested that this reaction resulted from a DDP-membrane interaction.

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