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ORIGINAL ARTICLE

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Differences in intracellular sites of action of Adriamycin in neoplastic and normal differentiated cells

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Abstract *Purpose*: This study was performed to clarify the intracellular specificity of the differential cytotoxic effects of Adriamycin (ADM) on neoplastic and normal cells. *Methods*: The mouse lymphocytic leukemia cell line L1210 and pig kidney proximal tubular epithelial cell line LLC-PK_1 were used as neoplastic and normal cells, respectively. These cells were treated with various concentrations of ADM for 24 h and toxicological parameters were determined. *Results*: ADM (0.1–10 μ*M*) significantly down-regulated cell growth rate and [^{3}H]thymidine incorporation into DNA in the log phase, and at concentrations of more than 1 μ*M* reduced the viability of both cell lines. Lipid peroxidation was increased at 1 μ*M* ADM in L1210 cells and at 10 μ*M* ADM in LLC-PK_1 cells. The microsomal and nuclear fractions of both cell lines showed approximately the same level of ADM-induced superoxide anion (O_2^-) production, while the mitochondrial fraction of differentiated LLC-PK_1 cells produced the highest levels of O_2^-. Differentiated LLC-PK_1 cells showed the highest mitochondrial NADH-cytochrome *c* reductase activity. L1210 cells showed lower mitochondrial activities of enzymes involved in scavenging of reactive oxygen species, such as superoxide dismutase, glutathione peroxidase and catalase, than the other cells. *Conclusions*: These results suggest that ADM exerts cytostatic effects on neoplastic and normal undifferentiated cells through the inhibition of DNA synthesis by DNA intercalation, and cytotoxic effects on neoplastic cells through the accumulation of reactive oxygen species resulting from low scavenger enzyme activities. The cytotoxic effects on normal differentiated cells may be related to the high levels of production of reactive oxygen species due to high mitochondrial NADH-cytochrome *c* reductase activity.

Key words Adriamycin · Microsomes · Mitochondria · Reactive oxygen species scavenging enzymes · Superoxide anion

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Introduction

Adriamycin (ADM) is an anthracycline antibiotic that exhibits excellent antitumor activity against a variety of solid tumors and hematological malignancies. Most of the adverse effects are reversible and are commonly seen with other antitumor agents. ADM, in a similar manner to other antitumor agents, exhibits serious toxic effects in tissues with rapid cell cycles such as myeloid and lymphatic tissues, intestinal mucosa, the testis and ovary. Cardiotoxicity is very specific and limits the total dose of ADM that may be administered since the effect is cumulative [23, 27]. No such dose-limiting cardiotoxic effects are observed with other antitumor agents [26].

Two mechanisms of intracellular cytotoxicity of ADM have been proposed: the production of reactive oxygen species by one-electron-reduced ADM [2, 15, 34], and the inhibition of DNA synthesis by intercalation into the DNA [14, 43] or inhibition of topoisomerase II activity [13, 36]. It has been reported that the cardiotoxicity of a series of related anthracycline antitumor agents can be dissociated from their antitumor activity [10]. Furthermore, the amount of ADM that accumulates in cells is not closely related to its cytotoxicity [22, 38]. Thus, we speculated that the organ-specific toxicity of ADM would be determined either by the inhibition of DNA synthesis or the production of radicals. This study was performed to clarify the reason for the differential effects of ADM on undifferentiated neoplastic cells and differentiated normal cells, assuming that the inhibition of DNA synthesis may have a cytostatic effect and that the production of reactive oxygen species may be linked to the cytotoxicity.

Materials and methods

Reagents

ADM hydrochloride, epinephrine, cytochrome *c* (from horse heart, type IV), catalase (Cat, bovine liver), and superoxide dismutase (SOD, bovine erythrocytes) were obtained from Sigma Chemical Company (St. Louis, Mo.). NADH and NADPH were obtained from Boehringer Mannheim (Mannheim, Germany). [methyl-^{3}H]Thymidine (75 Ci/mmol) was from the Radiochemical Center, Amersham (Tokyo, Japan). Other chemicals and solvents used were of analytical grade.

Cell lines and culture

The mouse lymphocytic leukemia cell line L1210 (American Type Culture Collection, CCL 219, passage 379) was obtained from Riken Cell Bank (Tsukuba, Japan). The cells were maintained in 15 m*M* HEPES-buffered (pH 7.3) Dulbecco's modified Eagle's medium containing 5% fetal calf serum (Gibco, Grand Island, N.Y.), seeded at a density of 1×10^5 cells/ml and incubated at 37 °C in an atmosphere of 5% CO_2 in air. L1210 cells were used in the log phase of growth after 2 days in culture as undifferentiated neoplastic cells.

The pig kidney proximal tubular epithelial cell line LLC-PK$_1$ (American Type Culture Collection, CRL 1392, passage 196) was obtained from Flow Laboratories (Rockville, Md.). The cells were maintained in 15 m*M* HEPES-buffered (pH 7.3) Dulbecco's modified Eagle's medium/Ham's F12 medium (1:1) containing growth supplements [40] and 5% fetal calf serum, seeded at a density of 1×10^5 cells/ml and incubated at 37 °C in an atmosphere of 5% CO_2 in air. LLC-PK$_1$ cells were used at subconfluency after 2 days and at confluency after 4 days in culture as undifferentiated and differentiated normal cells, respectively.

Cells were treated with various concentrations of ADM for 24 h and toxicological parameters were determined.

Growth inhibition and cytotoxicity

L1210 cells in culture were counted and their viability determined before and after the addition of ADM by trypan blue dye exclusion using a hemocytometer. Aliquots of the cell suspension were centrifuged (1000 *g*, 5 min, 4 °C) and the supernatant was collected for lactate dehydrogenase (LDH) assay. LLC-PK$_1$ cells were fixed and stained by adding a solution of methylene blue (5 g/l in 50% ethanol) and the cells were counted before and after the addition of ADM calculated by the method of Graeme et al. [19]. The culture supernatant was collected for LDH assay. LDH released from cells of each cell line into the culture medium during cultivation was measured by the method of Wroblewski and LaDue [42].

Peroxidized lipid content

The peroxidized lipid contents of L1210 and LLC-PK$_1$ cells were measured as the production of malondialdehyde (MDA) as described by Ohkawa et al. [32]. Cells were washed twice with ice-cold phosphate-buffered saline (PBS). The cell pellets were treated with 1 ml of *N*/12 sulfuric acid and 0.125 ml 10% phosphotungstic acid and then centrifuged. The pellet fraction and 0.067% 2-thiobarbituric acid solution were heated in a boiling water bath for 1 h and allowed to cool. The MDA formed was then extracted with 1.5 ml *n*-butanol by vortex mixing for 30 s. Following centrifugation, the organic extract was collected. The fluorescence intensity of each extract was measured by spectrofluorometry with excitation and emission wavelengths of 515 and 553 nm, respectively. 1,1,3,3-Tetraethoxypropane was used as a standard for this assay.

DNA synthesis

Each cell line was seeded and incubated as described above. Various concentrations of ADM were added to the cells followed by incubation for 3 h. Then, [methyl-^{3}H]thymidine (0.5 μCi/dish) was added followed by incubation for a further 3 h. The cells were washed with ice-cold PBS, 5% trichloroacetic acid and methanol. The acid-insoluble fraction was dissolved in 0.1 *N* NaOH and the radioactivity was measured by liquid scintillation counting.

O_2^- production and reactive oxygen species scavenger enzyme activity in the nuclear, mitochondrial and microsomal fractions of different cells

L1210 cells in log phase and LLC-PK$_1$ cells at both subconfluency and confluency were harvested and washed three times with ice-cold PBS by centrifugation (1000 *g*, 5 min, 4 °C). The cell pellets were homogenized in 50 m*M* Tris–HCl (pH 7.4) buffer containing 0.25 *M* sucrose and 1 m*M* EDTA. The homogenate was centrifuged for 10 min at 600 *g* to obtain the nuclear pellet. The supernatant was centrifuged for 20 min at 10,000 *g* to obtain the mitochondrial pellet. The supernatant was centrifuged for 1 h at 105,000 *g* to obtain the microsomal pellet. Nuclear, mitochondrial and microsomal pellets were washed with the above homogenization solution and then resuspended and sonicated in 0.1 *M* potassium phosphate buffer (pH 7.5) to obtain a final protein concentration of 10 mg/ml as determined by the method of Bradford [6] using a Bio-Rad protein assay kit (Bio-Rad Laboratories, Richmond, Calif.).

O_2^- production by the nuclear, mitochondrial and microsomal fractions was determined by the adrenochrome formation assay [8] by measuring the absorption change at 485 nm ($\epsilon = 2.96$/m*M* per cm) and 30 °C. The reaction mixture consisted of the enzyme, 1 m*M* epinephrine, 0.35 m*M* NADH (for the mitochondrial fraction) or NADPH (for the nuclear and microsomal fractions) and 0.1 m*M* ADM in 0.1 *M* potassium phosphate buffer (pH 7.5).

The mitochondrial NADH-cytochrome *c* reductase (Cyt-Red) activity was measured according to the method of Mahler [24]. The nuclear and microsomal NADPH-cytochrome P450 reductase (P450-Red) activities were determined as described by Omura and Takesue [33]. Glutathione peroxidase (GSH-Px) and Cat activities were assayed according to the methods of Carmagnol et al. [9] and Decker [12], respectively. SOD activity was determined from the oxidation rate of epinephrine to adrenochrome according to the method of Misra and Fridovich [28].

Statistical analysis

Statistical analysis was performed with Dunnett's multiple comparison test.

Results

Cell growth inhibition and cytotoxicity in different cells

ADM at concentrations of 0.1 to 10 μ*M* significantly reduced both the cell growth rate (Fig. 1A) and [^{3}H]thymidine incorporation into DNA (Fig. 1C) in the log phase in L1210 cells. ADM at concentrations of more than 1 μ*M* reduced the number and viability of the cells, but at 0.1 μ*M* had no effect on cell viability (Fig. 2A). ADM at 0.1 to 10 μ*M* also significantly reduced the cell growth rate (Fig. 1B) and [^{3}H]thymidine incorporation into DNA (Fig. 1C) in the log phase in LLC-PK$_1$ cells. ADM at the low concentration of 0.1 μ*M* inhibited [^{3}H]thymidine incorporation in L1210 cells to a greater extent than in differentiated LLC-PK$_1$ cells (Fig. 1C).

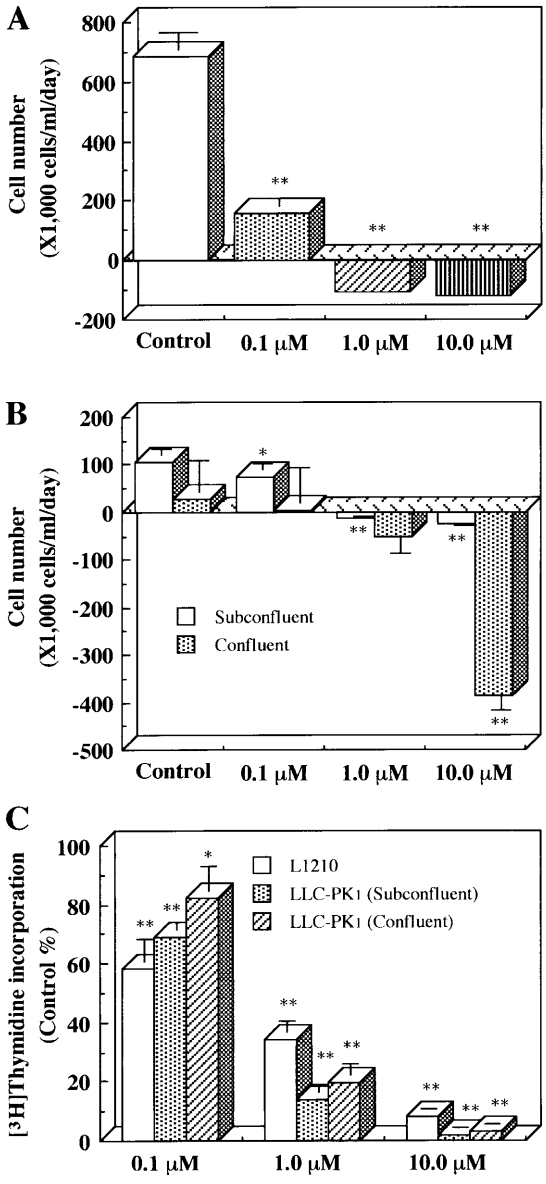

Fig. 1A–C Effects of ADM on the cell growth of L1210 and LLC-PK_1 cells. **A** L1210 cells in log phase were treated with various concentrations of ADM for 24 h. The numbers of cells were determined before and after the addition of ADM based on the number of trypan blue dye-excluding cells counted using a hemocytometer. The numbers of cells were calculated from the difference between the values obtained before and after the addition of ADM. Each column represents the mean ± SD of three cultures. **B** LLC-PK_1 cells at subconfluency (undifferentiated) and confluency (differentiated) were treated with various concentrations of ADM for 24 h. The numbers of cells were determined before and after the addition of ADM based on the concentration of methylene blue dye. The numbers of cells were calculated from the difference between the values obtained before and after the addition of ADM. Each column represents the mean ± SD of three cultures. **C** L1210 and LLC-PK_1 cells were treated with various concentrations of ADM for 3 h, and labeled with [^{3}H]thymidine for 3 h. The amounts of [^{3}H]thymidine incorporated into the DNA of untreated L1210 cells and LLC-PK_1 cells at both subconfluency and confluency were 4,295,000 ± 206,000, 875,000 ± 5,500 and 166,300 ± 7,000 dpm per milligram protein per hour, respectively. The values shown in the columns are percentages of control and are the means ± SD of three cultures. $*P < 0.05$, $**P < 0.01$, vs control

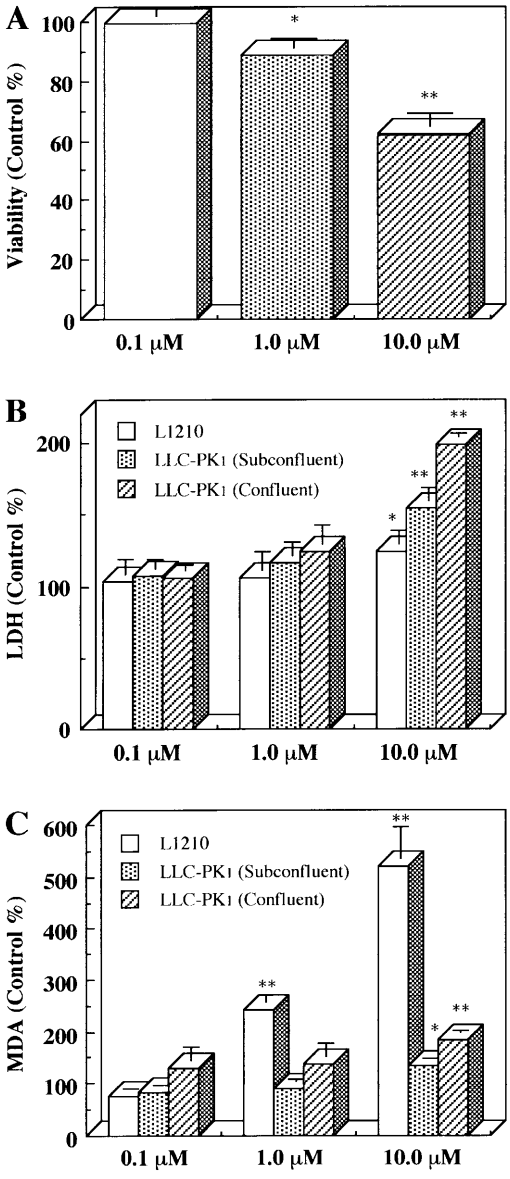

Fig. 2A–C Effects of ADM on the cytotoxicity of L1210 and LLC-PK_1 cells. **A** Effects of ADM on the viability of L1210 cells. The cell viability is expressed as the percentage of trypan blue dye-excluding cells. **B** Leakage of LDH from L1210 and LLC-PK_1 cells induced by ADM. **C** Production of MDA by L1210 and LLC-PK_1 cells induced by ADM. The experimental protocols for the treatment of L1210 and LLC-PK_1 cells with ADM are described in the legend for Fig. 1. The cell viability of untreated L1210 cells was 96.9 ± 0.2%. LDH released into the culture medium from untreated L1210 cells and LLC-PK_1 cells at both subconfluency and confluency were 6.5 ± 0.4, 11.8 ± 0.3 and 21.7 ± 0.1 IU, respectively. The contents of MDA in untreated L1210 cells and LLC-PK_1 cells at both subconfluency and confluency were 172.7 ± 5.2, 537.4 ± 13.4 and 625.6 ± 54.5 picomoles per milligram protein, respectively. The values shown in the columns are percentages of control and are the means ± SD of three cultures. $*P < 0.05$, $**P < 0.01$, vs control

The number of undifferentiated and differentiated LLC-PK_1 cells was decreased by 1 and 10 μ*M* ADM (Fig. 1B). The high concentration of 10 μ*M* caused a much greater decrease in numbers of differentiated LLC-

PK_1 cells than of L1210 cells or undifferentiated LLC-PK_1 cells. High concentrations of ADM caused the leakage of LDH from cells of both cell lines, and this effect was most pronounced in differentiated LLC-PK_1 cells (Fig. 2B).

Superoxide production and reactive oxygen species scavenging activities in different cells

The peroxidized lipid content was significantly increased with 1 μ*M* ADM in L1210 cells and with 10 μ*M* in undifferentiated and differentiated LLC-PK_1 cells (Fig. 2C). ADM at the high concentration of 10 μ*M* caused a greater increase in the peroxidized lipid content in L1210 cells than in the other cells.

There were no significant differences in activity of ADM-induced O_2^- production in the presence of NADPH by the microsomal and nuclear fractions of the different cell lines (Fig. 3). Nuclear ADM-induced O_2^- production and P450-Red activity were lower than those of the other organelle fractions (Fig. 3). Differentiated LLC-PK_1 cells showed higher levels of ADM-induced O_2^- production in the presence of NADH in the mitochondrial fraction, of Cyt-Red in the mitochondrial fraction and of P450-Red in the microsomal fraction than the other cells (Fig. 3). L1210 cells had lower activities of Cyt-Red in the mitochondrial fraction, of P450-Red in the microsomal fraction (Fig. 3) and of reactive oxygen species scavenger enzymes such as SOD, GSH-Px and Cat in the mitochondrial fraction than the other cells (Fig. 4).

Discussion

The differential cytostatic and cytocidal effects of ADM between undifferentiated neoplastic and differentiated normal cells were studied. ADM at lower concentrations reduced cell growth rate and DNA synthesis in the log phase in L1210 and LLC-PK_1 cells without associated decreases in cell viability. These effects of ADM were more marked in neoplastic L1210 cells. Many studies have demonstrated that ADM is selectively stored in the nuclei of a variety of tissues and cells [5, 17, 20, 25]. These observations suggest that ADM is transferred into the nucleus and intercalates into the DNA [14, 43] or

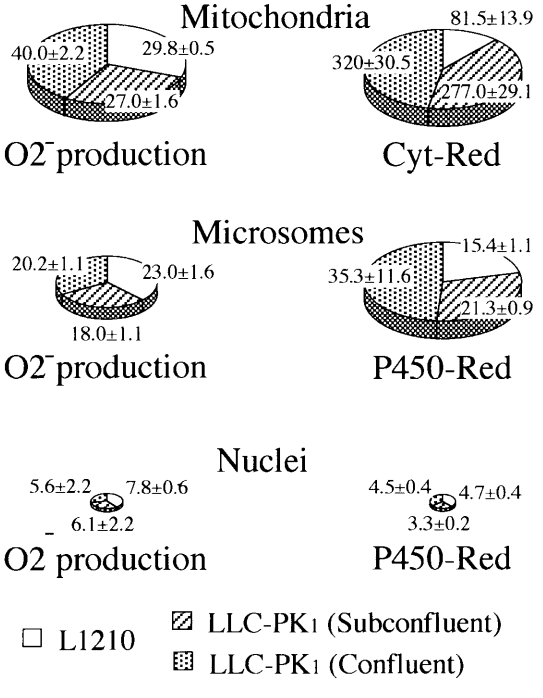

Fig. 3 Production of O_2^- by ADM and activities of related enzymes in the organelles of L1210 and LLC-PK_1 cells. O_2^- production is shown as nanomoles of adrenochrome formed per milligram protein per minute. Cyt-Red and P450-Red activities are shown as nanomoles ferricytochrome *c* reduced per milligram protein per minute. Each value is the mean ± SD of three to five experiments. The sizes of the pie segments labeled *O2⁻ production* by the microsomal and nuclear fractions indicate percentages in relation to the respective mitochondrial fractions. The sizes of the pie segments labeled *P450-Red* in the nuclear fraction indicate percentages in relation to the respective microsomal fractions. Each pie segment represents the mean value

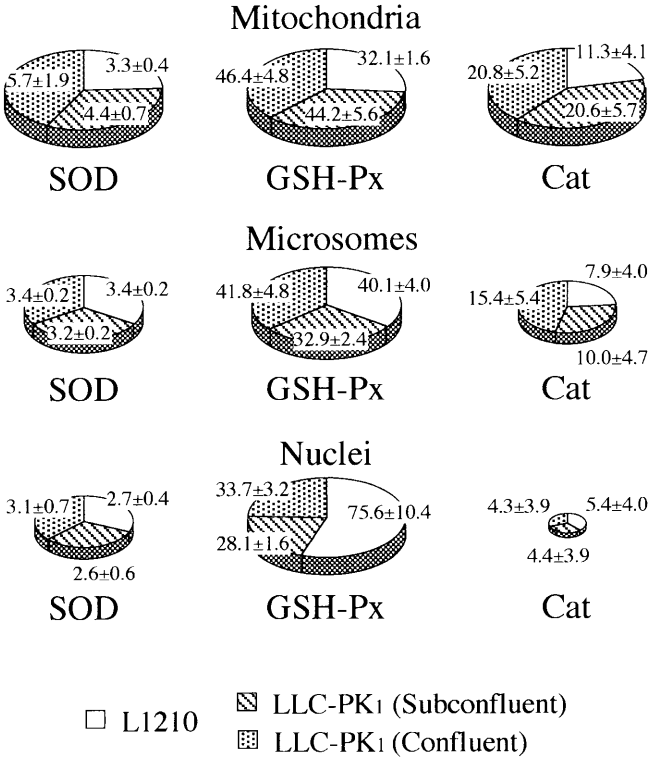

Fig. 4 Activities of reactive oxygen species scavenging enzymes in the organelles of L1210 and LLC-PK_1 cells. SOD activity is shown as activity units per milligram protein per minute. GSH-Px activity is shown as nanomoles NADPH oxidized per milligram protein per minute. Cat activity is shown as millimoles hydrogen peroxide scavenged per milligram protein per minute. Each value is the mean ± SD of three to five experiments. The sizes of the pie segments for each enzyme activity in the microsomal and nuclear fractions indicate percentages in relation to the respective mitochondrial fractions. Each pie segment represents the mean value

inhibits topoisomerase II activity [13, 36], and thus shows a cytostatic effect on neoplastic and normal undifferentiated cells due to inhibition of DNA synthesis. The level of uptake of ADM by L1210 cells is higher than that by LLC-PK_1 cells (unpublished observation), indicating that ADM exerts a stronger cytostatic effect on the former. Thus, it is likely that ADM-induced inhibition of DNA synthesis has a cytostatic effect on neoplastic and normal undifferentiated cells, and that this effect would be distinguishable from the cytocidal effect of ADM applied at lower concentrations.

Several investigations of the mechanism of ADM-induced cytotoxicity have suggested that free radical reactions and lipid peroxidation may play a role in the cytocidal effect of ADM [2, 15, 34]. In this study, ADM at a higher concentration induced increases in lipid peroxidation in L1210 and LLC-PK_1 cells. In addition, ADM-induced oxygen radical production has been shown to occur in other cells, for example in platelets [39], hepatocytes [1], heart [37] and Ehrlich tumor cells [16]. Many compounds that can cause cell death are metabolized to electrophilic free radicals which is followed by the production of reactive oxygen species. ADM is also metabolized to ADM semiquinone radical intermediate by microsomal [3, 41] or nuclear [4] P450-Red and mitochondrial Cyt-Red [15], and this intermediate reacts with molecular oxygen to form O_2^- and other reactive oxygen species [21]. The present study showed that the levels of ADM-induced O_2^- production by the mitochondrial fractions of L1210 and LLC-PK_1 cells were higher than those of the microsomal and nuclear fractions.

The cardiotoxicity of ADM is characterized by swelling and disruption of the mitochondrial membranes [18]. Appreciable amounts of ADM are also distributed in the mitochondria of cardiac and tumor cells and cells of other organs [7, 11, 30]. The reduction of ADM to free radicals by mitochondrial Cyt-Red [15] following mitochondrial localization of ADM may cause the mitochondrial toxicity. The ADM-induced mitochondrial toxicity due to reactive oxygen species may contribute to the cytocidal effect of ADM not only in cardiac cells but also in other types of cells that possess appreciable levels of ADM and higher levels of mitochondrial ADM-induced O_2^- production. Thus, the most significant cytocidal effect of ADM at the high concentration on differentiated LLC-PK_1 cells may be related to the high levels of production of reactive oxygen species due to the high mitochondrial Cyt-Red activity.

Generally, tumor cells have lower activities of reactive oxygen species scavenging enzymes such as SOD, GSH-Px and Cat than normal cells [29, 31, 35]. In this study, we also found lower levels of these enzyme activities in the mitochondria of L1210 cells. Thus, it is likely that ADM exerts cytocidal effects on neoplastic cells through the accumulation of reactive oxygen species due to the lower activities of radical scavenging enzymes.

In conclusion, our findings suggest that the differential effects of ADM between neoplastic and normal cells are determined by the cellular characteristics. That is, DNA synthesis inhibition would cause the cytostatic effect and the reactive oxygen species production would be related to the cytocidal effect. ADM at low concentrations would be transferred into the nucleus and exert a cytostatic effect on neoplastic and normal undifferentiated cells through the inhibition of DNA synthesis. At higher concentrations, ADM would show a cytocidal effect on neoplastic cells due to accumulation of reactive oxygen species resulting from the low activities of scavenging enzymes. On the other hand, the cytotoxic effect of ADM at a high concentration on normal differentiated cells would be related to the high levels of production of reactive oxygen species due to the high levels of mitochondrial Cyt-Red activity.

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