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Prevention of Hydrogen Peroxide and Cisplatin Induced Apoptosis by Intracellular Catalase Overexpression

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Abstract: Apoptosis induced by hydrogen peroxide (H_2O_2) was investigated in Chinese hamster ovarian (CHO) cells, and compared to apoptosis induced by cisplatin (25 µM), a well-established apoptosis-inducing agent. In preliminary experiments, CHO cells were treated with low concentrations of H_2O_2 (250-500 µM) continuously or for 3 hours, followed by washing and further incubation in complete growth medium for 1-10 days. Fluorescence microscopy of acridine orange-stained cells indicated morphological changes of apoptosis. Similar changes were observed in cisplatin-treated cells. DNA-laddering also revealed that DNA fragmentation occurred in both adherent and non-adherent cells by day 2. To prevent H_2O_2-mediated apoptosis, the catalase gene was isolated, subcloned into a mammalian expression vector (pCDM8) and transfected into CHO cells. Transiently transfected cells contained approximately 8-fold increased catalase levels. Overexpression of intracellular catalase provided protection for the CHO cells from H_2O_2 as measured by a tetrazolium-formazan reduction assay. Furthermore, cisplatin-mediated apoptosis was prevented by intracellular catalase overexpression, indicating that this cytotoxic drug acts at least in part through an H_2O_2-dependent pathway.

Key Words: Apoptosis, catalase, cisplatin, hydrogen peroxide, transfection.

Hücreiçi Katalaz'ın Arttırılması ile Hidrojen Peroksid ve Sisplatin ile İndüklenen Apoptoz'un Önlenmesi

Özet: Hidrojen peroksid (H_2O_2) ile indüklenen apoptoz Chinese Hamster Ovarian (CHO) hücrelerinde araştırıldı ve iyi bilinen bir apoptoz indükleyici ajan olan sisplatin (25 µM) ile karşılaştırıldı. İlk deneylerde, CHO hücreleri H_2O_2'in düşük konsantrasyonları (250-500 µM) ile devamlı olarak veya 3 saat inkübasyonu takiben yıkanıp kültür ortamına alınarak 1-10 gün süreyle inkübe edildi. Akridin oranj ile boyanmış hücrerin floresan mikroskopi ile incelenmesi sonucunda apoptozda görülen morfolojik değişiklikler saptandı. Benzer değişiklikler sisplatin ile muamele edilen hücrelerde gözlendi.

This study was carried out in Departments of Immunology and Cardiovascular Medicine at Imperial College School of Medicçine, London: ENGLAND.

DNA elektroforezi de DNA fragmentasyonunun hem aderan hem de aderan olmayan hücrelerde 2 güne kadar başladığını ortaya koydu. H_2O_2 ile oluşturulan apoptozu önlemek için, katalaz geni izole edildi, bir memeli ekspresyon vektörüne (pCDM8) klonlandı ve transfekte edildi. Geçici olarak transfekte edlien hücrelerinin yaklaşık 8-kat daha fazla katalaz içerdikleri saptandı. Katalazın intrasellüler olarak arttırılmış ekspresyonu tetrazolium-formazan redüksiyon deneyi ile gösterildiği gibi CHO hücrelerini H_2O_2'den korudu. Ayrıca, cisplatin ile oluşturulan apoptoz da, arttırılmış intrasellüler katalaz ekspresyonu ile engellendi. Bu veri ayna zamanda bu sitotoksik ilacın en azından bir kısım etkisinin H_2O_2'e bağımlı bir yolla oluştuğunu düşündürmektedir.

Anahtar Sözcükler: Apoptoz, katalaz, sisplatin, hidrojen peroksid, transfeksiyon.

Introduction

The complex biochemical changes that accompany cellular injury can result in cell death by one, or both, of two mechanisms, termed necrosis and apoptosis (1). Although the morphological and biochemical changes seen in these two types of cell death are quite distinct, in vivo both mechanisms may occur in the same pathological process. For example, inflammatory mediators produced or induced by necrotic cells may cause apoptosis in surrounding cells, such as vascular endothelial cells. However, cell injury does not always cause cell death, since a number of mechanisms permit cells to survive.

The interaction of reactive oxygen intermediates (ROI) with lipid, protein, carbohydrate and DNA molecules in cells causes cell injury, and eventually can lead to cell death, either from necrosis or apoptosis. Antioxidant mechanisms act to limit cell injury, but oxidative damage is often not completely prevented. It may be more efficient in terms of energy consumption for a cell to replace or repair damaged molecules than to maintain high levels of anti-oxidants (2). Another possibility is that ROIs play significant physiological roles in inducing appropriate changes in cell function, such as stimulating cell proliferation, enhancing phagocytic defence mechanisms or activating the cells, for instance, through activation of the nuclear factor-kappa B (NF-κB) pathway (3). However, exaggerated increases in the levels of ROIs within the tissues are always deleterious.

Catalase, one of the most important antioxidant enzymes, was first described by Warburg in 1923 (4). This original study showed that catalase contains iron, and that it can be inhibited by cyanide. Catalase isolated from bovine liver was crystallised in 1937 by Sumner and Dounce, representing one of the earliest successful crystallisations of an intracellular enzyme (4). Catalase enzymes from different species share similarities in molecular weight, numbers of subunits and types of prosthetic groups (5).

Catalase is composed of four identical subunits. Each subunit of this tetramer is approximately 60 kDa in molecular weight and contains a single heme group, in which the iron is in the ferric state, and a strongly bound NADPH molecule (6). The iron-heme active site fits into a cleft of the protein structure, allowing only small proteins to gain access (7). This enzyme is therefore only involved in the reduction of small molecules, such as H_2O_2 and methyl or ethyl hydroperoxides, and does not metabolise large molecular peroxides, such as lipid hydroperoxides (8). It has a high reaction capacity, but low affinity for H_2O_2 (9). In aerobic cells, catalase is

predominantly found in peroxisomes, which contain enzymes generating H_2O_2 (9). The highest levels of catalase are found in hepatocytes, kidney and erythrocytes, whereas neuronal cells, myocytes, lung cells, endothelial cells, pancreatic cells and connective tissues have the lowest catalase contents (10-12).

The basic action of catalase is to catalyse the dismutation of H_2O_2 as follows:

$$2H_2O_2 \xrightarrow{\text{catalase}} 2H_2O + O_2$$

Catalase can be inactivated by O_2^{-}. . This event is prevented by superoxide dismutase (SOD) (13), providing the basis for a synergism between SOD and catalase.

The complete DNA and amino acid sequence of human catalase is now known (14). In addition, sequence analysis of 1.7 kb of the 5'-flanking region of the gene shows that human catalase exhibits features of a house keeping gene with no TATA box, a high GCC content, multiple CCAAT boxes, and a transcription start site (15).

In this study, we investigated whether the overexpression of intracellular catalase provides protection against the hydrogen peroxide- and cisplatin-induced apoptosis. We showed that catalase protects the cells from apoptosis induced by both hydrogen peroxide and cisplatin. That cisplatin-induced apoptosis can be prevented by catalase overexpression suggests that such gene-targeted strategies providing increased cellular antioxidant capacity could be used to protect normal cells, such as bone marrow, during chemotherapy for neoplastic diseases.

Materials and Methods

Cell Lines and Culture

Chinese hamster ovarian (CHO) cells were obtained from Dr. Martyn Robinson (Celltech, Slough, UK) as a gift, and maintained in Minimal Essential Medium (MEM) supplemented with 10% FCS, 2 mM L-Glutamine, 100 U/ml penicillin and 100 µg/ml streptomycin at 37°C and 5% CO_2.

Plasmid constructs. An empty plasmid (insertless) pCDM8 was obtained from Invitrogen (Leek, The Netherlands). The pCDM8/catalase construct to overexpress catalase under the transcriptional control of the CMV promoter has been described previously (16). QIAGEN™ plasmid purification kit (Qiagen, Hilden, Germany) was used for large scale preparations of these plasmids prior to transfections.

CHO cells were transfected in six-well tissue culture plates using cationic liposomes (LipofectAMINE, Gibco, Paisley, UK) as described previously (16). Experiments were carried out 48-72 hours following the start of transfection.

Measurement of catalase immunoreactivity. In order to measure the catalase levels using an immunoassay, a competitive ELISA was used, as described previously (16). Catalase levels were determined by reference to a standard curve made with serial dilutions of human catalase. Results were normalised to total protein concentrations determined using a modified Lowry

method (17). The assay was shown to be reproducible, and sensitive down to approximately 0.1 U of catalase.

Assessing cell morphology. CHO cells were treated with either 250 μM H_2O_2 or 25 μM cisplatin in Hanks' Balanced Salt Solution (HBSS) for 3 hours at 37°C. After this treatment, cells were rinsed with HBSS and incubated in fresh complete growth medium for 2 days. As a negative control, some cells were incubated with HBSS prior to incubation in complete medium. Following a 2-day culture, non-adherent and adherent cells (removed from the plastic with trypsin) were mixed and washed in PBS. The cell morphology was investigated by staining with acridine orange (4 μg/ml) prepared in PBS. Morphological changes were viewed in a fluorescence microscope (Nikon, Japan).

DNA fragmentation analysis. DNA fragmentation was analysed by the method of Herrmann et al. (18) with some modifications. In brief, cells were exposed to low concentrations of H_2O_2 (250-500 μM) or cisplatin (25 μM), as described above, or continuously in culture medium (2 x complete growth medium). The cells were then harvested and washed as above. Cell pellets were incubated for 10 seconds with 50 ml of lysis buffer (1% NP-40 in 20 mM EDTA, 50 mM Tris-HCl, pH 7.5) and then recentrifuged. The supernatants were collected, and the lysing step repeated. The supernatants were brought up to 1% SDS and 2.5 mg/ml proteinase K (Gibco), and incubated at 37°C overnight. After addition of 1/10 vol. 10 M NaCl, the DNA was precipitated with ethanol and resuspended in sterile distilled water. Following incubation with RNAse A (Pharmacia) at a final concentration of 5 mg/ml at 37°C for 2 hours, the samples were separated by electrophoresis in 1.5% agarose gel.

Cell viability and proliferation assay. Cells transfected with either pCDM8/catalase or pCDM8 were seeded to a density of 10^5 per well. The next day, the cells were treated with 250 μM H_2O_2 or 25 μM cisplatin in the presence or absence of catalase (1 U/ml) and incubated for several days (1-10) days. One day before the measurement of cell viability, untransfected CHO cells were titrated on parallel wells to allow the construction of a standard curve for the cell number. The cell number was assessed using a commercial tetrazolium-formazan reduction assay (MTS assay, Promega Corp., Madison, WI) according to the manufacturer's instructions. Cell numbers were determined from the standard curve, and % cell viability was determined by the equation:

$$\% \text{ viability} = [1-((A-B)/A)] \times 100$$

A= Cell number of untreated cells at day 1.

B= Cell number of treated cells at the day of measurement.

Statistical analysis. Students' t test was used to determine the significance between the groups. A *p* value of 0.05 was considered significant.

Results

Low concentrations of H_2O_2 induce apoptosis in CHO cells. To determine the mechanism of H_2O_2-mediated cell death, CHO cells were treated for 3 hours with H_2O_2 at various

concentrations (0-10 mM) and were subsequently incubated in complete growth medium. Morphological changes were determined at various times post-exposure (1-4 days) by staining non-adherent and adherent cells with acridine-orange, which binds to DNA. Cisplatin (25 μM) was used as a positive control for inducing apoptosis (19, 20).

Control CHO cells exposed to HBSS alone did not undergo any morphological change (Figure 1A). Cells treated with 250 μM H_2O_2 and incubated in complete medium for 2 days showed the characteristic features of apoptosis, including plasma membrane blebbing and nuclear fragmentation (Figure 1B). Similar changes occurred in cisplatin-treated cells (Figure 1C). In contrast to the effects of 250 μM, high concentrations (over 1 mM) of H_2O_2 caused immediate cell death with the morphological features of cell lysis (data not shown). These data suggest that exposure of cells to low concentrations of H_2O_2 causes programmed cell death, whereas H_2O_2 at high doses leads to necrotic death.

An analysis of DNA fragmentation was performed to confirm the conclusions obtained from the morphological study above. DNA extracted from H_2O_2– and cisplatin-treated CHO cells showed a characteristic internucleosomal ladder pattern on gel electrophoresis (Figure 2). DNA laddering was not seen with untreated cells (Figure 2, lane 2). This result confirms that the cells undergo programmed cell death in the presence of low concentrations of H_2O_2.

Overexpression of catalase in CHO cells. Chinese hamster ovarian (CHO) fibroblasts were transiently transfected with the pCDM8/catalase construct, which leads to the constitutive expression of the catalase gene under the control of the CMV promoter, following which levels of catalase immunoreactivity were measured by ELISA. Approximately 8-fold increased catalase levels were determined in CHO cells transiently transfected with the catalase gene (Figure 3).

Overexpression of catalase prevents cellular injury induced by long-term exposure to low concentrations of H_2O_2 . The effects of exposure of cells to H_2O_2 for periods of up to 10 days were tested using CHO cells transfected with pCDM8/catalase and measuring cell numbers with the MTS assay. Some mock transfected cells were also exposed to H_2O_2 in the presence of 1 U/ml catalase in order to compare the effects of overexpressing intracellular catalase with the effects of supplementing extracellular catalase. As shown in Figure 4, overexpression of intracellular catalase provided long-term protection for up to 10 days, whilst the protective effect of catalase added to the extracellular medium was restricted to 5 days. The reason for the limited protective effect of extracellular catalase addition may be due to the enzyme not being in the optimal subcellular location, or loss of activity of the enzyme as the medium was not changed during the experiment.

Overexpression of intracellular catalase by transfection with pCDM8/catalase also protected cells from long-term cisplatin-induced death (Figure 5). In this case, addition of exogenous catalase had no effect on long-term survival. Transfection of catalase alone had no effect on cell survival in the absence of H_2O_2 (data not shown). These data suggests that cisplatin may lead to target cell apoptosis by altering intracellular levels of ROIs, in particular H_2O_2. Addition of exogenous catalase is ineffective at blocking this pathway, presumably because it is unable to act in the intracellular site of increased H_2O_2.

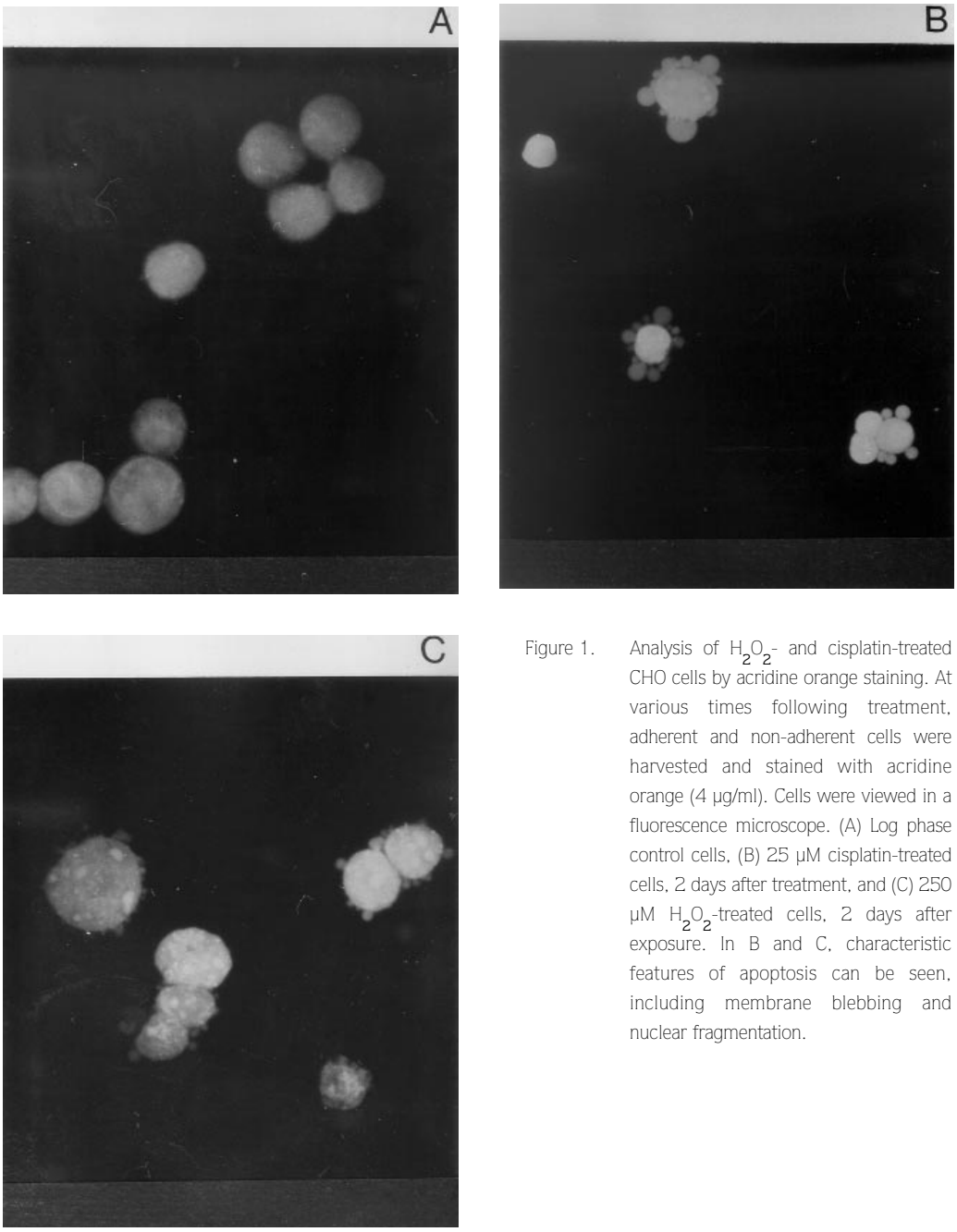

Figure 1. Analysis of H_2O_2- and cisplatin-treated CHO cells by acridine orange staining. At various times following treatment, adherent and non-adherent cells were harvested and stained with acridine orange (4 μg/ml). Cells were viewed in a fluorescence microscope. (A) Log phase control cells, (B) 25 μM cisplatin-treated cells, 2 days after treatment, and (C) 250 μM H_2O_2-treated cells, 2 days after exposure. In B and C, characteristic features of apoptosis can be seen, including membrane blebbing and nuclear fragmentation.

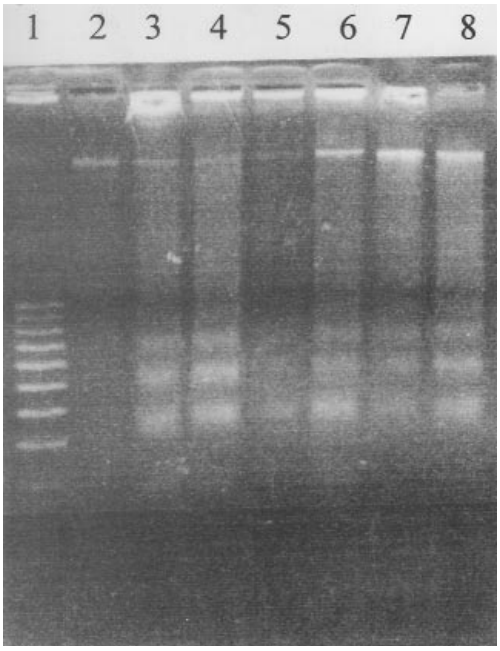

Figure 2. DNA fragmentation induced by H_2O_2 and cisplatin. Total DNA was isolated from both adherent and nonadherent CHO cells and run on a 1.5% agarose gel. *Lane 1*, 200bp DNA ladder, *Lane 2* control, log phase cells; 3, 5 and 7, at day 2 after 3 hours incubation; 4, 6, and 8, continuously incubation with 250 μM, 500 μM H_2O_2 and 25 μM cisplatin, respectively.

Discussion

Cell death occurs in two ways; *i.* apoptosis and *ii.* necrosis (1). ROIs cause cell death by interacting with protein, lipid, carbohydrate and DNA molecules (4). Oxidative stress is a mediator of apoptosis, since: *i.* apoptosis can be induced by the addition of ROIs or depletion of intracellular anti-oxidants, *ii.* agents inducing ROI production can mediate apoptosis, *iii.* the addition of anti-oxidants inhibits apoptosis (21-23). H_2O_2 is a critical molecule, as it can be converted to more toxic ROIs. It has been previously shown that H_2O_2 induces cell death in a dose- and time-dependent manner (23, 24). The present study demonstrated that low concentrations of H_2O_2 mediate apoptosis in the long term with characteristic morphological changes and nuclear fragmentation in CHO cells (Figure 1, 2). However, high doses of H_2O_2 led to cell death in a very short time period by cell necrosis (data not shown). Similar observations have been described for other cell types (25, 26). Several strategies to protect the cells from ROI-mediated injury and death have been studied. The use of anti-oxidants in chemical or pharmacological forms in vitro have shown that these are protective against injury due to their corresponding oxidants, such as catalase against H_2O_2 (23, 27). However, in practice, using these anti-oxidants in vivo is problematic. For instance, catalase has a very short half life in vivo (10-20 minutes) due to rapid renal clearance, it is unable to cross cell membranes, and it is sensitive to proteases (27-30). Thus, targeting genes for overexpressing anti-oxidants within the intracellular environment could be an alternative approach to overcoming this problem (16, 27).

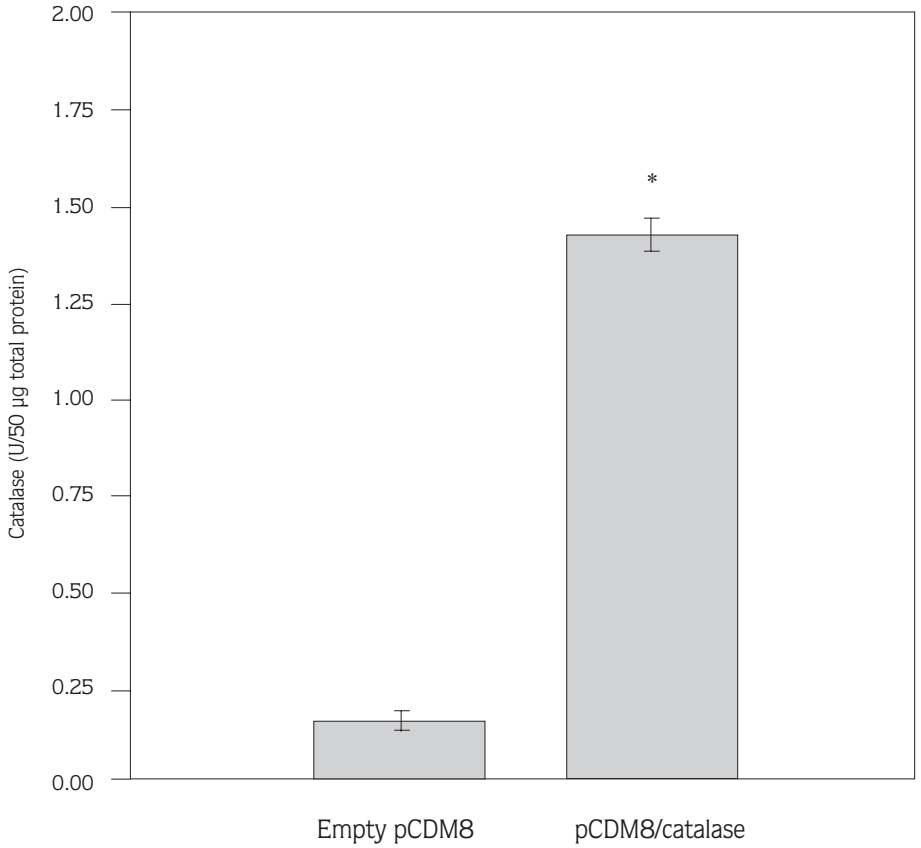

Figure 3. Catalase overexpression in transiently transfected CHO cells. The construct encoding catalase cDNA under the transcriptional control of CMV promoter (pCDM8/catalase) was transfected into CHO cells. The catalase levels were determined by ELISA, and the results presented in comparison to empty plasmid (pCDM8) transfected cells. Values represent mean ± SD of triplicate determinations. (*) $p<0.001$ versus empty pCDM8 transfected cells.

The intracellular catalase overexpression in transiently transfected CHO cells was protective against both apoptotic and necrotic cell death (Figure 4). The overexpression of intracellular catalase also prevented cisplatin-mediated cell death, suggesting that the cytotoxic effects of cisplatin may be mediated by altering intracellular ROI levels, such as H_2O_2 (Figure 5). A link between protection from ROIs and resistance to cisplatin has been previously suggested (31-33). Oxidant-resistant CHO cells were produced by chronic exposure (over 200 days) to progressively increasing concentrations of H_2O_2, and contained significantly higher catalase and total glutathione levels when compared to parental cells (31, 34). These cells also showed increased resistance to cisplatin-mediated cell death. In this study we used gene expression to

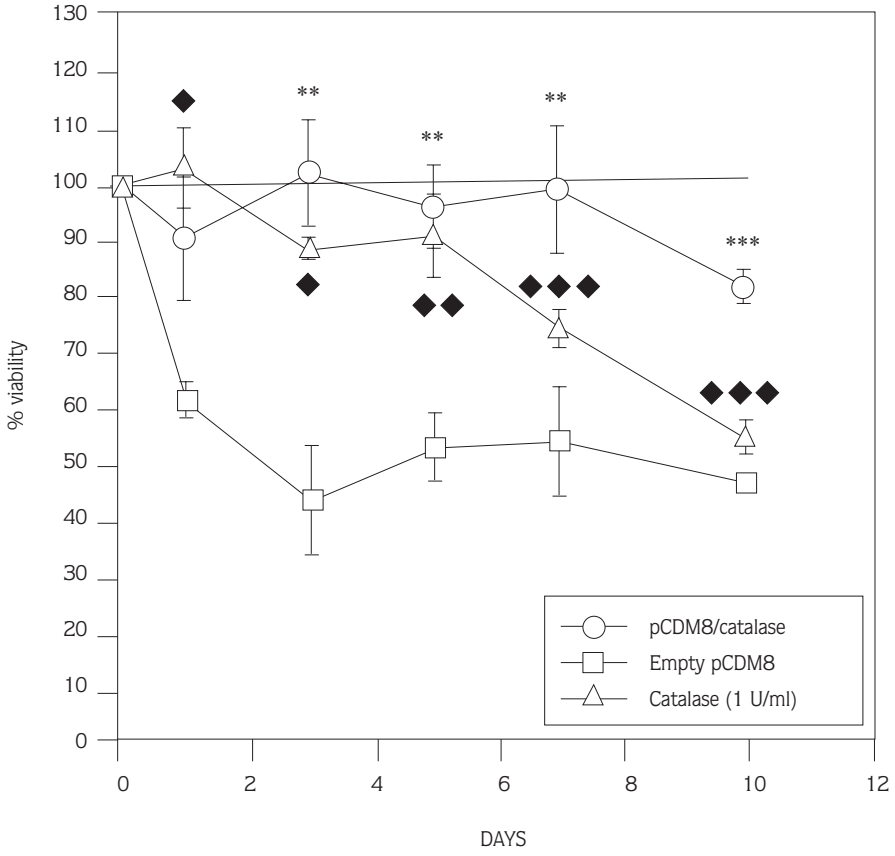

Figure 4. Effect of intracellular catalase overexpression on long term H_2O_2 exposure. The catalase gene transfected and empty plasmid (pCDM8) transfected CHO cells were exposed to 250 μM H_2O_2 for several days, following which cell viability was measured by the MTS assay. One set of empty plasmid transfected cells was exposed to H_2O_2 in the presence of catalase (1U/ml). Values are mean + SD of triplicates. (*) $p<0.02$, (**) $p<0.002$, (***) $p<0.001$; (◆) $p<0.002$, (◆◆) $p<0.005$, (◆◆◆) $p<0.02$ versus empty pCDM8 transfected cells. Stars and diamonds represent significance for pCDM8/catalase transfected and catalase treated cell groups, respectively.

demonstrate that overexpression of catalase is sufficient for protection against cisplatin, indicating the important role of H_2O_2 in cisplatin-induced apoptosis.

In conclusion, these data are important in helping determine the mechanisms of cisplatin-induced apoptosis. They also suggest that overexpression of catalase may be a useful strategy for protecting normal cells (in particular bone morrow) during chemotherapy for neoplastic disease.

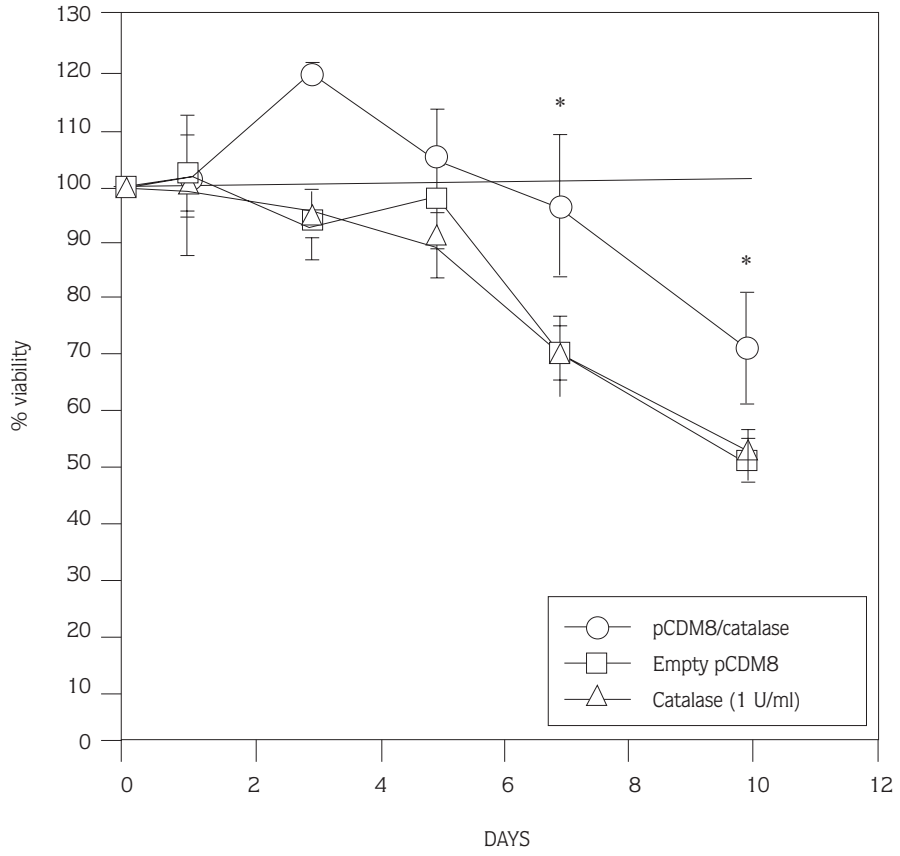

Figure 5. Effect of intracellular catalase overexpression on long-term cisplatin exposure. The catalase gene (pCDM8/catalase) transfected and empty plasmid (pCDM8) transfected-CHO cells were treated with 25 μM cisplatin for several days. Cell viability was measured by the MTS assay. One set of empty plasmid transfected cells was exposed to cisplatin in the presence of catalase (1 U/ml). Results are means ± SDs of triplicates. (*) $p<0.05$ versus empty pCDM8 transfected cell group.

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