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Effects of Oxidative Stress on Apoptosis and Antioxidant Enzyme Levels

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Abstract – Effects of oxidative stress on the induction of apoptosis and the activity of antioxidant enzymes were investigated in HL-60 cells using H_2O_2 and cisplatin which generate oxygen species in the cell. Various concentrations of oxidants were treated to cells and at different incubation time, cells were harvested for assays. Cell viability, morphology by propidium iodide staining and DNA fragmentation by agarose gel electrophoresis were observed to determine whether they induce apoptosis. The activity of antioxidant enzymes such as superoxide dismutase and catalase was also measured to evaluate the cellular response to the oxidative damage. The results are as follows: H_2O_2 induced apoptosis at 10 μM after 6h incubation, while it took 12h for cisplatin. Both oxidants induced the superoxide dismutase activity at a tolerable low concentration. However, at a concentration which causes apoptotic cell death, the enzyme level was dropped markedly at first and then recovered to the normal level after which it declined again, probably due to cell death. On the other hand, changes in the activity of catalase were not significant at most concentrations except the statistically significant decrease at 24h after 10 μM-H_2O_2 treatment. In this study, H_2O_2- and cisplatin-treated cells showed similar results in apoptotic response and enzyme activities, suggesting that anticancer activity of cisplatin may be related, at least in part, to the production of oxygen free radicals.

Keywords □ H_2O_2, cisplatin, apoptosis, superoxide dismutase, catalase

Reactive oxygen species such as O_2^-, H_2O_2, and OH^- can be produced in oxygen consumming organisms by oxygen metabolism and cells are likely to be exposed to these toxic by-products. These species are capable of severely damaging cells by oxidizing various components such as proteins and DNA, and causing chromosome aberrations, sister chromatid exchanges, single- and double-strand DNA breaks and lipid peroxidation (Ranjbar and Hannigan, 1993). For many years, these oxygen by-products have been suggested to be involved in many human diseases, such as cancer. Hydrogen peroxide is a strong oxidizing agent and known to have mutagenic (Ho and Crapo, 1987), clastogenic (Troy et al., 1990) and carcinogenic (Barber and Harris, 1994) activities. In the presence of reduced transition metals (McCord and Day, 1978), it generates the extremely destructive hydroxyl radical that attacks all cellular components. The radical is also formed when hydrogen peroxide reacts with superoxide. Cisplatin(cis-diaminedichloroplatinum (II)) is an active anticancer agent against several human cancers such as testicular, ovarian, cervical and bladder cancers. In aqueous solutions, it can be hydrolyzed to form both monoaquo and diaquo derivatives, which, in turn, can form various hydroxy species through deprotonation. Even though the high concentration of chloride in plasma prevents the hydrolysis of cisplatin, the low concentration in cells permits its hydrolysis (Foye, 1995), thereby exposing cells to oxidative stress.

The biological effects of oxidative stress on human cells are of particular interest due to its potential role in a number of diseases. In addition, depending on the individual susceptibility of tissues and the extent of oxidative load they receive, cells might respond in different ways to the stress. Recently, it has been reported that oxidative stress may cause cell death by apoptosis in various cells as a central protective response to excess oxidative damage (Ratan et al., 1994; Payne et al., 1995; Sanchez et al., 1996). Apoptosis, a programmed cell death, is a physiological event regulating the cell number or eliminating damaged cells in the processes like embryonic development and tumor growth. Activation of apoptosis leads to a series of biochemical and mor-

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phological events which result in nucleus disintegration and eventual fragmentation of the cell (Wyllie, 1980). When cells are attacked by oxidants, antioxidant enzymes such as superoxide dismutase (SOD) and catalase play an important role in dealing with free radicals. The SODs catalyze the conversion of the superoxide radical to H_2O_2, while catalase clear peroxide, preventing the production of hydorxyl radicals. Alterations in the expression of genes encoding these enzymes may occur in damaged cells to protect themselves or as a result of the response such as the induction of apoptosis (Camhi et al., 1995).

In this study, human myeloid leukemia HL-60 cells were treated with hydrogen peroxide and cell viability, the presence of internucleosomal DNA cleavage, a characteristic biochemical event in apoptosis, and the activity of antioxidant enzymes such as superoxide dismutase and catalase were determined. The experiments were carried out at various levels of damages and time courses to find out how cells respond to increasing amount of damages. In parallel studies, the effects of cisplatin, an anticancer drug that may produce free radicals, have also been investigated to compare and confirm its mechanism of action.

MATERIALS AND METHODS

Materials

Fetal calf serum, RPMI 1640 medium, and other reagents for cell culture were obtained from Gibco Laboratories (Grand Island, NY). RNase A, proteinase K, hydrogen peroxide, cisplatin, propidium iodide, epinephrine, and equilibrated phenol were purchased from Sigma Chemical Co. (St. Louis, MO). Bradford protein assay kit was from Bio-Rad Laboratories (Hercules, CA).

Cell Culture

The human myeloid leukemia HL-60 cells were maintained in log phase growth in T-25 flasks containing RPMI 1640 medium (pH 7.3) supplemented with 10% fetal calf serum, 100 IU/ml penicillin G, 100 μg/ml streptomycin, 10 mM HEPES and 2 mM L-glutamine. Suspension cultures were incubated at 37 ℃ in a humidified atmosphere of 5% CO_2. All cultures were passaged every 60h to maintain 10^5-10^6 cells/ml.

Chemical Treatments

a) H_2O_2 Treatments

In 6-well plates, HL-60 cells were suspended in PBS to the final cell number of 0.5×10^6 cells/ml and treated for 30 min in the presence of different concentrations (100 nM, 1 μM, 10 μM, 100 μM, 1 mM) of H_2O_2. Cells were resuspended in complete medium and incubated as usual. At each time point of 0 h, 6 h, 12 h, 18 h and 24 h, cells were harvested, washed twice with PBS and stored at -20 ℃ for assays.

b) Cisplatin Treatments

Cisplatin dissolved in DMSO was diluted with RPMI medium to a final concentration of 0.1% DMSO and used as a cisplatin stock solution. Different concentrations (100 nM, 1 μM, 10 μM, 100 μM, 1 mM) of cisplatin were added to HL-60 cells in 6-well plates and incubated in complete medium as usual. Cells were harvested at 1 h, 12 h and 24 h, washed and stored for assays.

Trypan Blue Exclusion

Viability of cells were measured by the Trypan Blue Exclusion method (Mosser and Martin, 1992). Cells in suspension were stained by adding an equal volume of 0.4% trypan blue and live cells were counted on hemocytometer.

Apoptotic Morphology

Untreated control and 10 μM of H_2O_2 or cisplatin treated cells (1×10^6) were harvested by centrifugation and fixed in cold ethanol with strong vortexing and kept at 4 ℃ overnight. Propidium iodide solution (ethanol: propidium iodide: RNase=3:1:6) was added to pellets after centrifugation and stained at least for 30 min (Bendirdjian et al., 1995). Images were photographed using Fluorescence Microscope.

DNA Extraction

DNA was extracted from cell pellets (10×10^6 cells) by lysing overnight at 51 ℃ in 0.5 ml lysis buffer (10 mM Tris-HCl pH 8.0, 1 mM EDTA, 10 mM NaCl and 1% SDS) and proteinase K (500 μg/ml). The lysed cells were extracted in equilibrated phenol, then treated with RNase A (80 μg/ml) at 37 ℃ for 45 min. The digests were extracted again with phenol : chloroform : isoamylalcohol (25 : 24 : 1) followed by chloroform: iso-amylalcohol (24 : 1). DNA was precipitated with 3M-sodium acetate and absolute ethanol and kept at -70 ℃ overnight. The pellets were washed with 70% ethanol after centrifugation, dried completely and then dissolved in TE buffer (10 mM Tris-HCl pH 8.0, 1 mM EDTA). The amount of DNA was measured at 260 nm and of protein at 280 nm (Yasuo et al., 1991).

Agarose Gel Electrophoresis

DNA(10 μg) was dissolved in loading buffer (0.25% BPB, 40% sucrose) and electrophoresed for 3 h at 80V

in 1.4% agarose gel containing ethidium bromide. TAE (40 mM Tris-acetate pH 8.0, 1 mM EDTA) was used as a running buffer and ECoRIHindIII (or HindIII alone) digests of λ DNA were used as a DNA size marker. The gels were photographed using UV Transilluminator.

Enzyme Assays

Superoxide Dismutase Activity-The adrenochrome assay (Misra and Fridovich, 1972) which determines the inhibition of epinephrine autoxidation, was used. In sodium carbonate buffer (pH 10.2), required volume of epinephrine stock solution was added to obtain a rate of increase in absorbance of 0.025/min. Cell pellets were sonicated in isotonic buffer and the amount of protein was measured by Bradford assay (Bradford, 1976). In each experiment, cell extract containing 10 μg of protein in 100 μl was added to this solution (1 ml) and the absorbance was measured at 295 nm using DU-68 Spectrophotometer. The per cent inhibition was calculated by using the following formula: % Inhibition of EP autoxidation

$=[\Delta A^{min-1}(control)-\Delta A^{min-1}(extract)]/\Delta A^{min-1}(control)$

Catalase Activity-The spectrophotometric assay (Beers and Sizer, 1952) was used. Hydrogen peroxide was added to 50 mM phosphate buffer, pH 7.0 to make 19 mM substrate solution. Cell extract (10 μg of protein/100 μl) prepared as above was added to this solution (1 ml) and the absorbance was measured at 240 nm. The conversion of the initial velocity to specific activity was calculated as follows: Specific Activity (units/mg)$=[\Delta A^{min-1}\times 1000]/[43.6\times$ mg protein/ml mix]

where 43.6 represents the molar extinction coefficient of peroxide. Protein concentrations were measured by Bradford assay.

RESULTS AND DISCUSSION

Common mechanism by which many oxidizing agents are known to cause cell injury is the increased production of oxygen free radicals. Hydrogen peroxide is a central molecule to produce free radicals and cisplatin may also produce free radicals by hydrolysis and deprotonation in cells. While free radicals may cause cellular damage in a variety of ways, in this work, we investigated their effects on the induction of apoptosis and influence on the levels of superoxide dismutase and catalase activity.

Cell Viability

Membrane integrity of the cells was monitored by their ability to exclude trypan blue dye. When low concentrations (100 nM and 1 μM) of H_2O_2 or cisplatin were treated, cells did not undergo a destructive pathway. However, at 10 μM, the survival was gradually decreased in both cases, only at a little slower rate in cisplatin treated cells. On the other hand, 100 μM treated cells showed drastic drop of survival to almost zero after 6 h incubation in H_2O_2 and 24 h in cisplatin treated cells (Fig.1).

Morphology by Propidium Iodide Staining

Morphologically, apoptosis is characterized by nuclear and cytoplasmic condensation of cells followed by loss of the nuclear membrane, fragmentation of the nuclear chromatin and subsequent formation of multiple fragments (Bendirdjian et al., 1995). In this study, 10 μM of H_2O_2- or cisplatin-treated cells showed nuclear chromatin

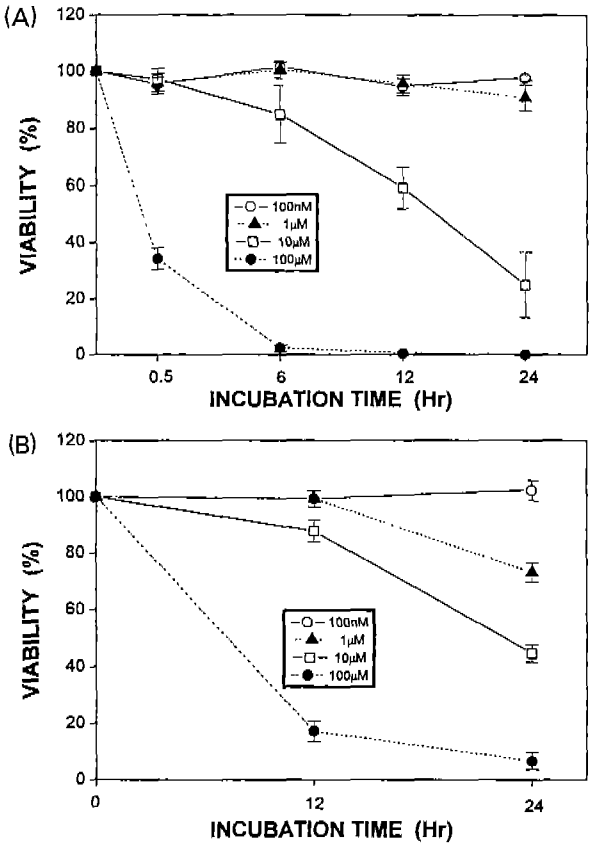

Fig. 1. Effects of hydrogen peroxide (A) and cisplatin (B) on survival of HL-60 cells. Cells were exposed to various concentrations of H_2O_2 and cisplatin for 30 min in PBS and then incubated in complete medium. Viability was assessed by adding an equal volume of 0.4% trypan blue to cells in suspension and viable cells were counted on hemocytometer. Data are the means of at least three independent determinations ± SD.

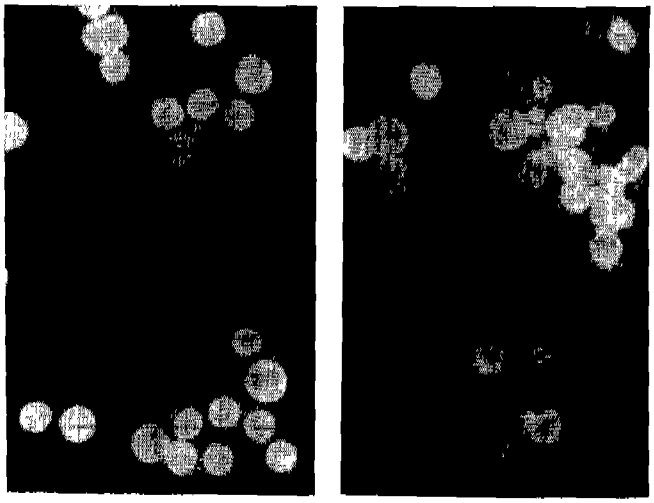

Fig. 2. Fluorescence Microscope appearance of untreated control (A) and 10 μM H_2O_2-treated (B) HL-60 cells. Cells were fixed with absolute ethanol, stained with propidium iodide for at least 30 min and observed under Fluorescence microscope (×400). The treated cells show overall condensation of chromatin around nuclear envelopes and discrete chromatin fragments.

condensation and resulting fragments were clearly visible under Fluorescence Microscope, while the untreated control cells exhibited no significant morphological change (Fig 2).

DNA Fragmentation by Apoptosis

Exposure to proper concentrations (10 μM and 100 μM) of H_2O_2 or cisplatin resulted in the characteristic ladder of DNA fragments on agarose gel electrophoresis stained by ethidium bromide. Such a ladder was not observed when the cells were exposed to the chemicals at concentrations less than 10 μM (Fig.3). In H_2O_2 treated cells, apoptotic fragments started to form after 6 h incubation at 10 μM, whereas it took 12 h in the case of cisplatin. Higher concentration and longer incubation caused mostly necrosis (data not shown), possibly because cells die instantaneously in response to an overwhelming insult. These results are agreeable, in part, with other reports that H_2O_2 (Yanagisawa-Shiota et al., 1995; Chiao et al.,1995) and cisplatin (Gorczyca et al., 1993) induce apoptosis. However, the conditions and cell types used in those experiments were different from present studies. Moreover, non of them showed the concentration and time dependent induction of apoptosis as clearly as the data shown here. This result confirms that cells induce apoptosis as a mechanism to cope with oxidative stress, but only when the level of damage is appropriate for the response.

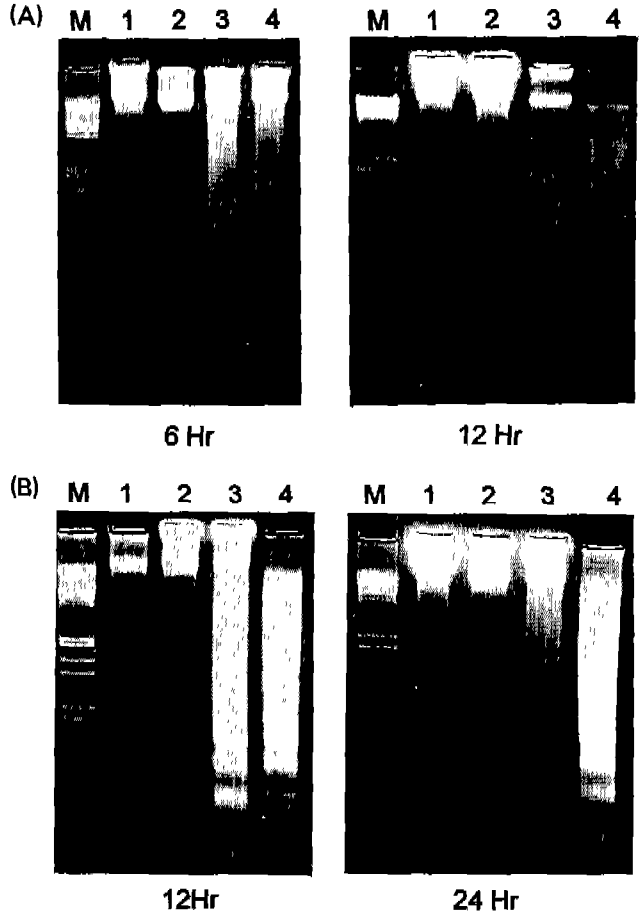

Fig. 3. Agarose gel electrophoresis of DNA extracted from H_2O_2 (A) and cisplatin (B) treated HL-60 cells. M: DNA size markers (ECoRIHindIII or HindIII alone); Lane 1: Control cells; Lane 2: 1 μM-treated cells; Lane 3: 10 μM-treated cells; Lane 4: 100 μM-treated cells. DNA was electrophoresed in 1.4% agarose gel containing ethidium bromide.

Superoxide Dismutase Activity

At a tolerable low concentration (100 μM) of H_2O_2 and cisplatin, the enzyme activity was increased significantly (Fig. 4). This may be due to the activation of SOD gene to protect the cell from injury. The degree of activation was much higher in cisplatin treated cells, suggesting that cisplatin is a stronger inducer of the enzyme. Since cells have protective mechanism against damage, this data implies that the normal protective function of the cell is working at this level of oxidative stress. However, at higher concentrations of H_2O_2 (1 μM and 10 μM) and cisplatin (10 μM), the SOD activity was first dropped markedly and then returned to normal level after which the activity was decreased again. The second drop

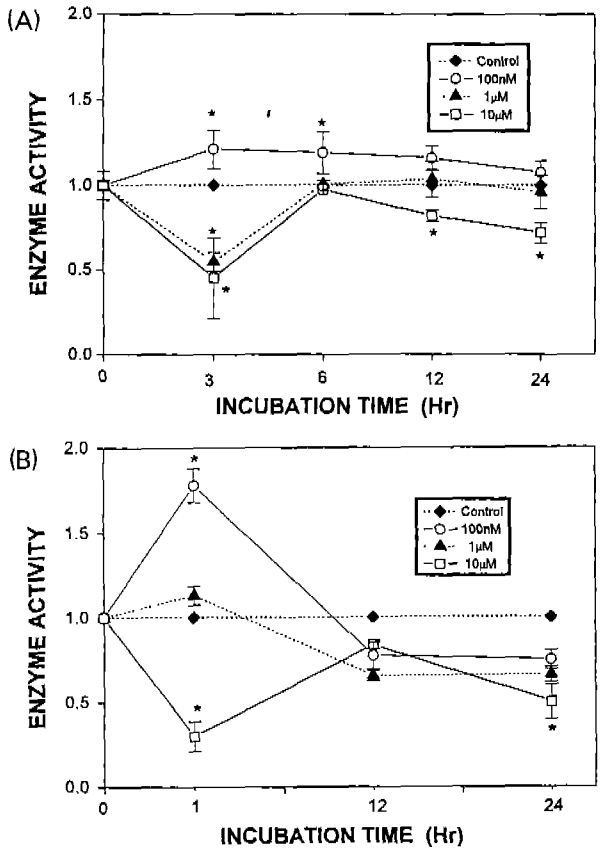

Fig. 4. Relative activity of superoxide dismutase in HL-60 cells treated with H_2O_2 (A) or cisplatin (B). The activity was determined by inhibition of spontaneous oxidation of epinephrine to adrenochrome. The increse of absorbance per min was measured and the enzyme activity was expressed as the ratio of % inhibition of treated to control cells.

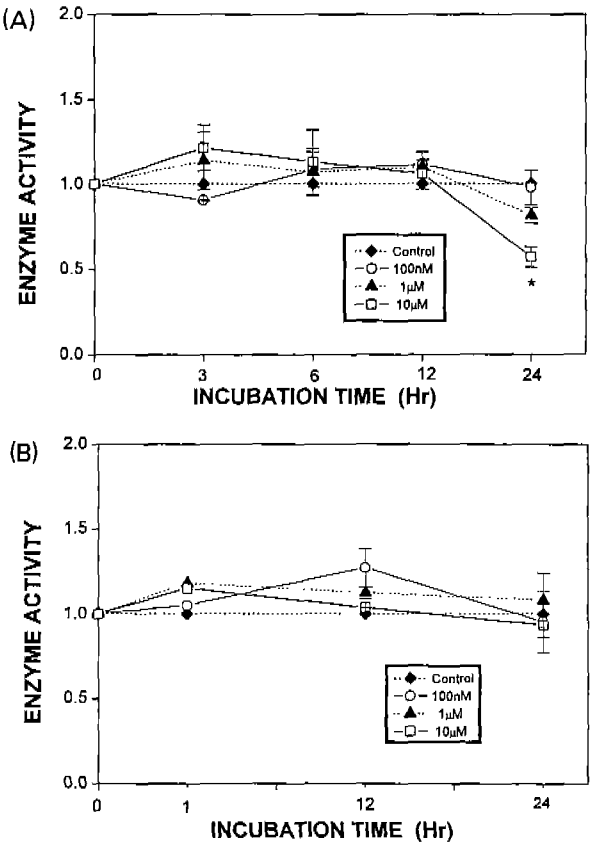

Fig. 5. Relative activity of catalase in HL-60 cells treated with H_2O_2 (A) or cisplatin (B). The activity was determined by a spectrophotometric method. The decrease of absorbance per min was measured and the enzyme activity was expressed as the ratio of specific activity of treated to control cells.

of the activity is probably due to the apoptotic cell death. At these concentrations, cells seem to receive damages exceeding the capacity of the cell to protect itself, which eventually ends up with apoptotic death. This concentration dependent opposite response of the cell may be explained by considering the different levels of stress cells receive prior to up or down regulation of SOD gene.

While SOD activity was reported to increase in cells exposed to heat or ethanol (Costa et al.,1993), in drug resistant cells (Mimnaugh et al.,1989; Batist et a.,1986) and during environmental stress (Herouart et al.,1993), other studies showed that the activity did not change at various experimental conditions (Ramu et al.,1984; Wiese et al.,1995). Our data may clarify the contradicting reports by demonsrating the concentration and time dependent response of the cell to different levels of oxidative stress. It may be noteworthy that the induction of apoptosis is coincide with the decrease of SOD activity. While cells are able to protect themselves by producing the enzyme, they seem to be in normal growth state. However, when the damage is exceeding to that level, SOD gene may be down regulated and cells start to die by apoptosis.

Catalase Activity

Fig. 5 illustrates small, non-significant increase of catalase activity at all concentrations tested in both H_2O_2- and cisplatin-treated cells. Even though there are reports that catalase avtivity is increased under certain conditions (Spitz et al.,1993), most reports present the results of no increase or even slight decrease of the activity (Batist et al.,1986). Wiese et al. (1995) also reported no significant increase in transcription and translation level of catalase gene in H_2O_2-adapted cells. Our data agrees with these reports that cells in oxidative stress do not activate genes for catalase production. Cells may undergo adaptation to the stressed state but it may not be through the induction of catalase, as demonstrated

in our study. In addition, catalase activity seems to have no relationship with the induction of apoptosis.

Our data suggest that there may be a correlation between the SOD activity and the induction of apoptosis, and also demonstrate the similarities between apoptotic response and enzyme activities that cells exert under oxidative stress caused by H_2O_2 and cisplatin.

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REFERENCES

Barber, D. A. and Harris, S. R. (1994). Oxygen free radicals and antioxidants: A review. *American Pharmacy.* **NS34(9)**, 26-35.

Batist, G., Tultule, A., Shinha, B. K., Katki, A., Myers, C. E. and Cowan, K. H. (1986). Overexpression of a novel anionic glutathione transferase in multidrug resistant human breast cancer cells. *J. Biol.Chem.* **261**, 15544-15549.

Beers, R. F. and Sizer, I. W. (1952). A spectrophotometric method for measuring the breakdown of hydrogen peroxide by catalase. *J. Biol.Chem.* **195**, 133.

Bendirdjian, E. S. and Sablon, A. J. (1995). Cisplatin resistance in a murine leukemia cell line is associated with a defective apoptotic process. *Exp. Cell Res.* **218**, 201-212.

Bradford, M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* **72**, 248-254.

Camhi, S. L., Lee, P. and Choi, A. M. (1995). The Oxidative stress response. *New Horiz* **3**, 170-182.

Chiao, C., Carothers, A. M., Grunberger, D., Solomon, G., Preston, G. A. and Barrett, J. C. (1995). Apoptosis and altered redox state induced by caffeic acid phenethyl ester in transformed rat fibroblast cells. *Cancer Res.* **55**, 3576-83.

Costa, V., Reis, E., Quintanilha, A. and Moradas-Ferreira, P. (1993). Acquisition of ethanol tolerance in Saccharomyces cerevisiae: the key role of the mitochondrial superoxide dismutase. *Arch. Biochem. Biophys.* **300**, 608-14.

Foye, W. O. (1995). Cancer Chemotherapeutic agents. pp122-123, ACE Professional Reference Book, Washington D. C. Gorczyca, W., Gong, J., Ardelt, B., Traganos, F. and Darzynkiewicz, Z. (1993). The cell cycle related differences in susceptibility of HL-60 cells to apoptosis induced by various antitumor agents. *Cancer Res.* **53**, 3186-92.

Herouart, D., Van Montagu M. and Inze, D. (1993). Redox-activated expression of the cytosolic copper/zinc superoxide dismutase gene in Nicotiana. *Proc. Natl. Acad. Sci. USA.* **90**, 3108-12.

Ho, Y. S. and Crapo, J. D. (1987). cDNA and deduced amino acid sequence of rat copper-zinc-containing superoxide dismutase. *Nucleic Acids Res.* **15**, 6746.

Mimnaugh, E. G., Dusne, L., Atwell, J. and Myers, C. E. (1989). Differential oxygen radical susceptability of Adriamycin-sensitive and -resistant MCF-7 human breast tumor cells. *Cancer Res.* **49**, 8-15.

Misra, H. P. and Fridovich,I. (1972). The role of superoxide anion in the autoxidation of epinephrine and a simple assay for superoxide dismutase. *J. Biol.Chem.* **247(10)**, 3170-3175.

Mosser, D. D. and Martin, L. H. (1992). Induced thermotolerance to apoptosis in a human T lymphocyte cell line. *J. Cell.Physiol.* **151**, 561-570.

Payne, C. M., Bernstein, C. and Bernstein, H. (1995). Apoptosis overview emphasizing the role of oxidative stress, DNA damage and signal transduction pathways. *Leuk Lymphoma* **19**, 43-93.

Ramu, A., Cohen, L. and glaubiger, D. (1984). Oxygen radical detoxification Enzymes in Doxorubicin-sensitive and -resistant P388 Murine Leukemia Cells. *Cancer Res.* **44**, 1976-1980.

Ranjbar, S. and Hannigan, B. M. (1993). DNA damage in human T-lymphoblastoma cell line Molt-3 induced by reactive oxygen species. *Mutat. Res.* **285**, 225-228.

Ratan, R. R., Murphy, T. H. and Barabam, J. M. (1994). Oxidative stress induces apoptosis in embryonic cortical neurons. *J. Neurochem.* **62**, 376-379.

Sanchez, A., Alvarez, A .M., Benito, M. and Fabregat, I. (1996). Apoptosis induced by tansforming growth factor-beta in fetal hepatocyte primary cultures: involvement of reactive oxygen intermediates. *J. Biol.Chem.* **27**, 7416-22.

Takano, Y. S., Harmon, B. V. and Kerr, J. F. R. (1991). Apoptosis induced by mild hyperthermia in human and murine tumour cell lines. *J. Pathology.* **163**, 329-336.

Troy, C. M., Muna, N. A., Greene, L. A., Price, D. L. and Shelanski, M. L. (1990). Regulation of peripherin and neurofilament expression in regenerating rat motor neurons. *Brain Res.* **529**, 232-238.

Wiese, A. G., Pacifici, R. E. and Davis, K. J. (1995). Transient adaptation to oxidative stress in mammalian cells. *Arch. Biochem. Biophys.* **318**, 231-240.

Wyllie, A. H. (1980). Glucocorticoid-induced thymocyte apoptosis is associated with endogenous endonuclease activation. *Nature* **284**, 555-556.

Yanagisawa-Shiota, F., Sakagami, H., Kuribayashi, N., Iida, M., Sakagami, T. and Takeda, M. (1995). Endonuclease activity and induction of DNA fragmentation in human mylogenous leukemic cell lines. *Anticancer Res.* **15**, 259-265.