J. Exp. Biomed. Sci. 11 (2005) 281-287

Expression of Bcl-2 and Caspase-3 Proteins Related to Apoptosis in Human Leukemia K-562 Cells

Jeong-Hyun Chang[1] and Heun-Young Kwon[2†]

[1]Department of Biology, Dong-A University, Busan 604-714, Korea.
[2]Department of Clinical Laboratory Science, College of Health Sciences, Catholic University of Pusan, Busan 609-757, Korea

Although actinomycin D (AMD) is known to induce apoptotic cell death to various cell lines, the mechanism of apoptosis induced by AMD is still unclear. Understanding this mechanism may improve its therapeutic efficacy. The present study has been performed to elucidate expression of Bcl-2 and Caspase-3 proteins related to apoptosis in human leukemia K-562 cells. Five different assays were performed in this study; DNA fragmentation analysis by agarose gel electrophoresis, quantitative assay of fragmented DNA, morphological assessment of apoptotic cells, quantification of apoptosis by annexin V (AV) and propidium iodide (PI) staning, and expression of Bcl-2 and Caspase-3 proteins by the western blot analysis. The number of apoptotic cells and amount of fragmented DNA in this cell line treated with AMD was increased at 6 hour. DNA ladder pattern was also appeared at 6 hour. The expression of Bcl-2 was decreased, and disappeared from 12 hours after AMD treatment. Precursor of Caspase-3 was degraded, and 20 kDa cleavage products were detected. These results suggest that AMD induced apoptosis of K-562 cells is Caspase-3-dependent fashion, and this apoptosis is related to the degradation of Bcl-2 proteins.

Key Words: Apoptosis, K-562 cells, Annexin V, Propidium iodide staning, Bcl-2, Caspase-3

INTRODUCTION

Appropriate cell number and organ size in a multicellular organism are determined by coordinated cell growth, proliferation and apoptosis. Disruption of these processes can cause cancer. Cells under normal conditions undergo apoptosis when exposed to a variety of cytotoxic agents for anticancer therapies (Barry et al., 1990; Lennon et al., 1991). Apoptosis is a normal physiological process in cell death characterized by cytoplasmic blebbing, cell shrinkage, nuclear condensation and DNA fragmentation prior to cell death. Dead cells are rapidly degraded by neighboring cells or macrophage (Cohen, 1992). Morphological changes of apoptosis are associated with double strand cleavage of nuclear DNA at the linker regions between nucleosomes. The resulting oligonucleosomal fragments can be detected as a ladder by agarose gel electrophoresis (Wyllie et al., 1984; Arends et al., 1990).

Actinomycin D (AMD), antineoplastic antibiotics, is a potent inducer of apoptosis in many cell lines. It was also shown to suppress programmed cell death in the PC12 (pheochromocytoma cell line) cells induced by etoposide (Batistatou et al., 1993; Martin et al., 1993). AMD induced apoptosis and Poly(ADP-ribose) polymerase-1 proteolysis in Hep-2 cells (Soldani et al., 2001), and increases caspase activity in a Drosophila neuronal cell line (Nagano et al., 2000).

The oncogenic protein Bcl-2 which is expressed in membranes of different subcellular organelles protects cells from apoptosis induced by endogenic stimuli (Rudner et al., 2002). Abnormal gene expression of Bcl-2 that inhibits apoptosis is found in a wide variety of human cancers. It contributes to the resistance of tumor cells to conventional therapies through interfering cell death signals triggered by chemotherapeutic agents (Liu et al., 2001). Caspase-3 has been

*Received: May 24, 2005
Accepted after revision: September 10, 2005
†Corresponding author: Heun-Young Kwon, Department of Clinical Laboratory Science, College of Health Sciences, Catholic University of Pusan, Busan 609-757, Korea.
Tel: 016-794-7273, Fax: 051-510-0569
e-mail: jhchang@donga.ac.kr

studied the most intensively, which is activated proteolytically when cells are signalled to undergo apoptosis (Yuan et al., 1996). Several substrates of Caspase-3 have been demonstrated, including PARP. The purpose of this study is to elucidate expression of Bcl-2 and Caspase-3 proteins related to apoptosis in K-562 cells.

MATERIALS AND METHODS

1. Cell culture

Human leukemia K-562 cells were used throughout this investigation. Suspension cultures of this cell line were grown at 37℃ in humidified 5% CO_2 incubator using Dulbecco's modified eagle medium (DMEM, GIBCO) supplemented with 10% newborn calf serum and gentamycin (50 μg/ml).

2. AMD treatment

AMD was dissolved in the serum free medium prior to use and exposed to cells at 37℃ for desired times. Cells were cultured for more than 12 hours in tissue culture petridishes prior to AMD the cells washed three times with phosphate buffered saline (PBS, pH 7.4).

3. Morphological assessment of apoptosis

Nuclear morphology was assessed by fluorescent microscopy after Hoechst 33258 staining. Five hundred microliter of cell suspensions ($5\times10^5 \sim 1\times10^6$/ml) were incubated with 5 μl Hoechst 33258 (1 mg/ml in ddH_2O) for 10 minutes. The samples were taken to microcentrifuge tubes and were centrifuged at 500 rpm for 2 minutes. After air-drying, coverslips were placed over cells to reduce light diffraction and 300~400 cells were counted in a fluorescence microscope equipped with a DAPI filter.

4. Quantitative and DNA fragmentation analysis

The apoptotic nature of cells was examined by agarose gel electrophoresis of their nuclear DNA, using the method of Waring (1990). The quantification of DNA fragmentation was carried as described by McConkey et al. (1989) with slight modifications. Cells treated with apoptosis inducer in a 100 mm culture dish were lysed in 0.33 ml of buffer containing 5 mM Tris, pH 8.0, 20 mM ethylenediaminetetraacetic acid (EDTA) and 0.5% Triton X-100. After incubation for 15 minutes on ice, samples were centrifuged for 10 minutes at 10,000 rpm to separate the intact chromatin (pellet) from the fragmented DNA (supernatant). Pellets were resuspended in 0.33 ml of a buffer containing 10 mM Tris, pH 8.0 and 1 mM EDTA. Pellet and supernatant fractions were separately assayed for DNA content using the diphenylamine reagent containing 1.5% diphenylamine, 1.5% sulfuric acid and 0.008% acetaldehyde in glacial acetic acid. DNA fragmentation was quantified by measuring the ratio of the DNA content in supernatant fraction to the total DNA content (supernatant plus pellet). For visualization of fragmented DNA, the supernatant fraction containing fragmented DNA was extracted two times with phenol and once with chloroform. Extracted DNA fragments was precipitated in 67% ethanol, 0.3 M sodium acetate at -70℃ for overnight, and then resuspended in a buffer containing 10 mM Tris, pH 8.0, 1 mM EDTA and 30 μg/ml RNase, prior to electrophoresis in 1.8% agarose gel as described by Jones et al. (1989).

5. Quantification of apoptosis by annexin V (AV) and propidium iodide (PI) staning

The Annexin V-FITC apoptosis detection kit (BD PharMingen) was used to measure apoptosis (Vermes et al., 2001). Cells were harvested by centrifugation at 2,000 rpm and the pellet was washed with cold PBS. Washed cells were resuspended in the binding buffer and stained with both AV-FITC and PI, according to the manufacturer's protocol, and analyzed by flow cytometry (FACScan, Becton Dickinson, San Jose, CA, USA). Cells unaffected by apoptosis and/or necrosis were defined as AV-/PI- (lower left quadrant). Early apoptotic cells were defined as AVþ/PI- (lower right quadrant), whereas late apoptotic and necrotic cells were defined as AVþ/PIþ (upper right quadrant). Finally, necrotic cells were defined as AV-/PIþ (upper left quadrant).

6. Western Blot analysis of Bcl-2 and Caspase-3 proteins

Cells were washed three times in cold PBS. Samples were then diluted with an equal volume of 2× SDS sample buffer and heated for 5 minutes at 100℃. Samples were loaded to equivalent amount (30 μg/lane) on one-dimensional SDS-polyacrylamide gel and subjected to electrophoresis. After electrophoresis, western blot analysis was done according to the technique of Towbin et al. (1979)

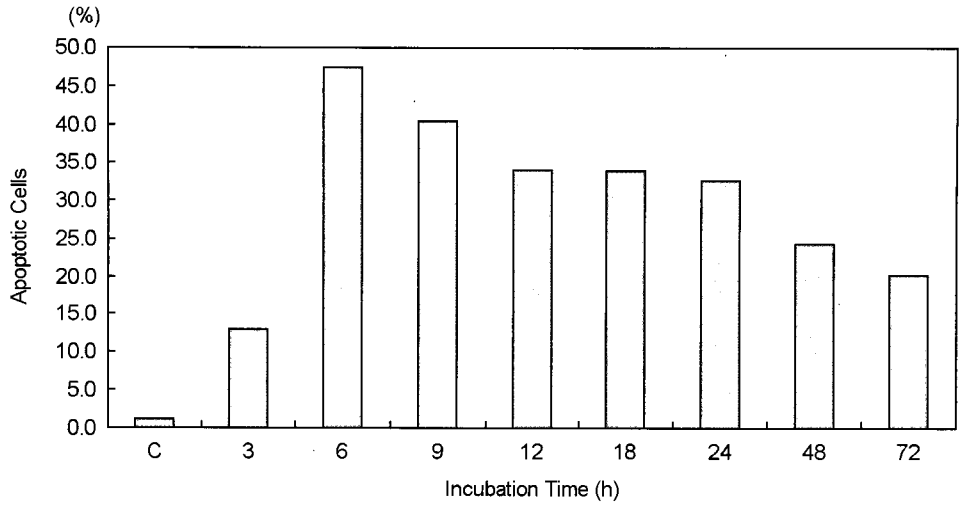

Fig. 1. Percentage of apoptotic cells assay in K-562 cells. Cells were treated with AMD, followed by incubation for 3, 6, 9, 12, 18, 24, 48 and 72 hours. The level of apoptosis was determined using fluorescence microscopy as described in methods and expressed as percentage of apoptotic cells. At least 400 cells were scored in each treatment.

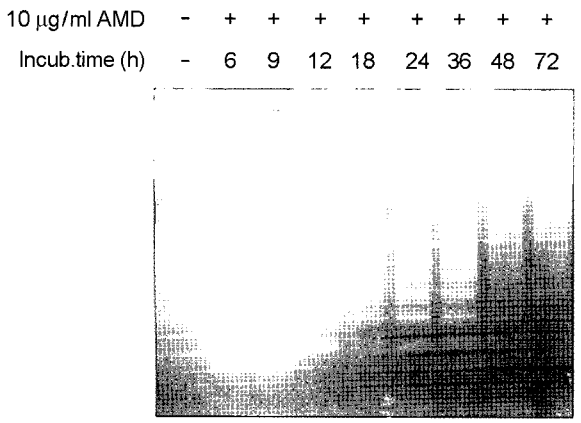

Fig. 2. DNA fragmentation pattern during apoptosis induced by AMD in K-562 cells. After treatment with AMD, cells were incubated for 6, 9, 12, 18, 24, 36, 48 and 72 hours at 37℃. Nuclear DNA was extracted with phenol and analyzed by 1.8% agarose gel electrophoresis.

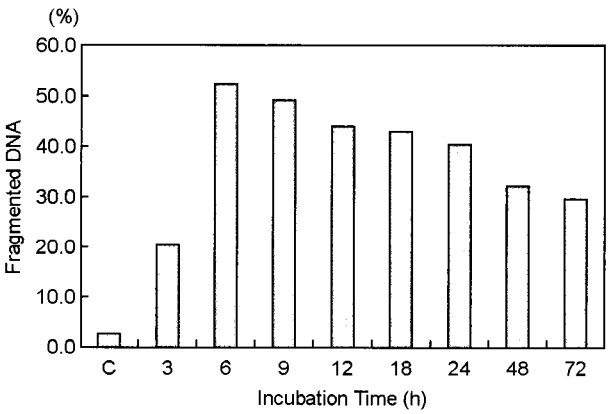

Fig. 3. Quantitative assay of fragmented DNA during apoptosis induced by AMD, in K-562 cells. After treatment with AMD, the cells were incubated for 6, 9, 12, 18, 24, 36, 48 and 72 hours at 37℃.

with slight modifications. Membrane was soaked in methanol for 10 seconds and washed in distilled water for 5 minutes. Blotting was performed at 0.35 A for 1 hour. And the membrane was dried by air drying. The membrane was treated with primary antibody (monoclonal anti-Bcl-2 and Caspase-3 proteins) for 1 hour, washed three times with PBS-Triton-X 100 (PBST). That was treated with secondary antibody (polyclonal anti-mouse/rabbit) was for 1 hour, followed by three times with PBST. The membrane was treated with Enhanced Chemileuminescence Liquid (ECL), and expose to X-ray film.

RESULTS

1. Apoptosis induced by actinomycin D

The percentage of apoptotic cells in K-562 cells treated with actinomycin D (AMD) is shown in Fig. 1. Hoechst 33258 stains nuclei of both live and dead cells blue when examined by fluorescence microscopy. Untreated normal cells showed homogenous staining of their nuclei. Apoptotic cells treated with AMD represented irregular staining of their nuclei as a result of chromatin condensation and nuclear fragmentation. The percentage of apoptotic cells in cells treated with 10 μg/ml AMD continuously increased up

to 12 hours after incubation and then gradually decreased.

DNA fragmentation during apoptosis in cells treated with AMD is shown in Fig. 2. After treatment with 10 μg/ml AMD, cells were incubated for various times. DNA ladder patten treated with AMD was shown at 12 hour.

Quantitative assay of fragmented DNA in cells is shown in Fig. 3. The amount of nucleosomal DNA fragment in cells treated with 10 μg/ml AMD most increased at 12 hour incubation and then gradually decreased as the incubation time.

Quantification of apoptosis by annexin V (AV) and propidium iodide (PI) staning in cells is shown in Fig. 4. Treatment with 10 μg/ml AMD induced apoptosis in K-562 cells. As showed in the FACS plots (left panel), low-dose treatment (B) induces cell death through apoptosis, demonstrated by the increased numbers of AV single positive cells (lower right quadrant), without changes in the numbers of PI single positive cells (upper left quadrant), compared to control treatment (A). High-dose treatment (C), on the other hand, induces cell death through both apoptosis and necro-

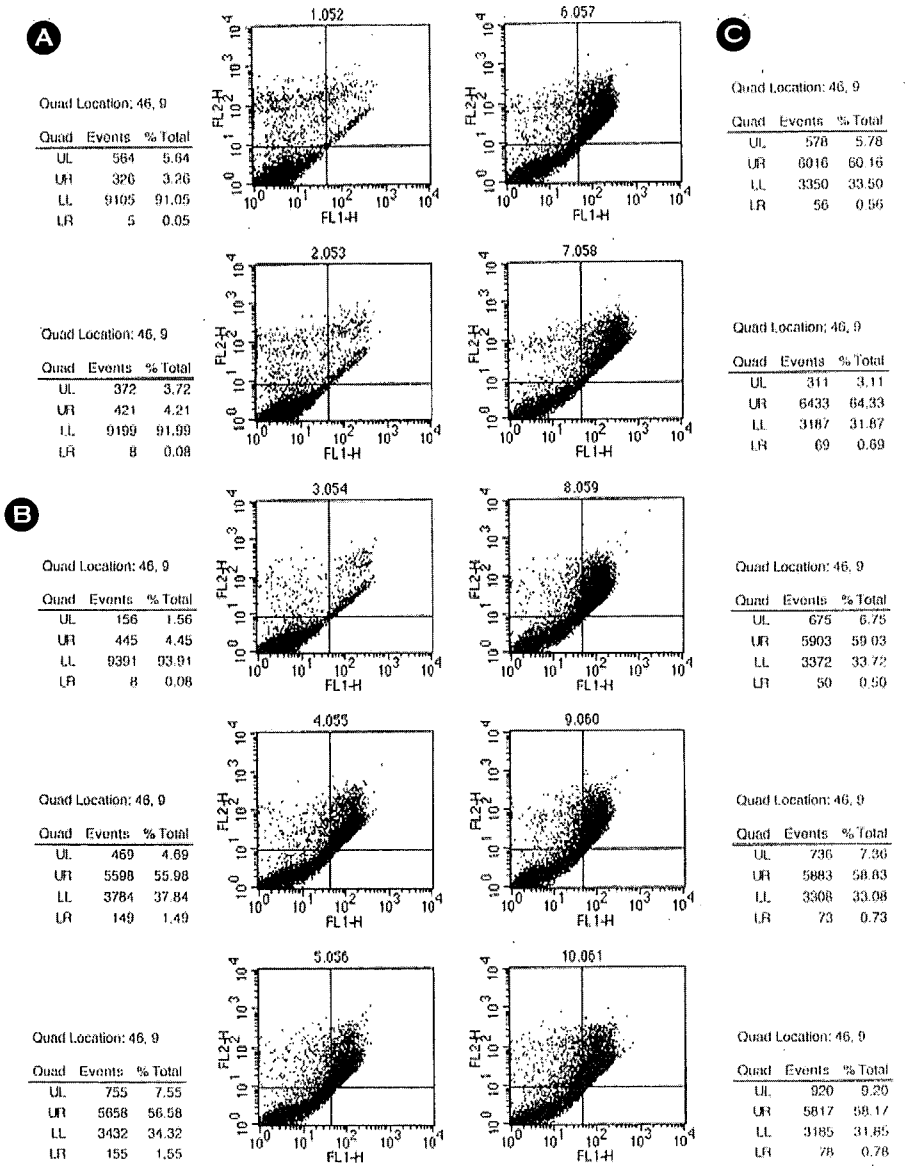

Fig. 4. Treatment with 10 μg/ml AMD induced apoptosis in K-562 cells. As showed in the FACS plots (left panel), low-dose treatment **(B)** induces cell death through apoptosis, demonstrated by the increased numbers of AV single positive cells (lower right quadrant), without changes in the numbers of PI single positive cells (upper left quadrant), compared to control treatment **(A)**. High-dose treatment **(C)**, on the other hand, induces cell death through both apoptosis and necrosis, demonstrated by the increase in the number of both AV single positive and PI single positive cells. Double positive cells (upper right quadrant) represent late apoptotic and necrotic cells. The immunofluorescent detection of DNA breaks by the TUNEL technique (right panel) demonstrates a dose-dependent increase of the number of apoptotic cells.

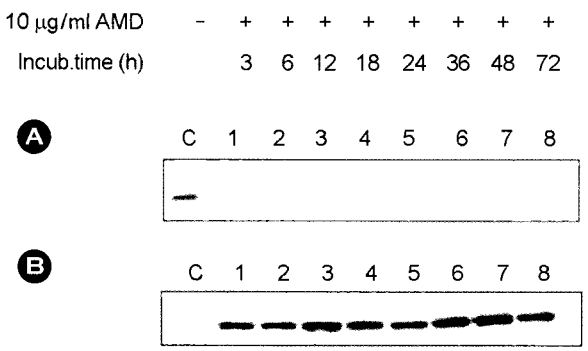

Fig. 5. Western blot analysis of Bcl-2 **(A)** and Caspase-3 **(B)** expression in K-562 cells. Cells were treated with AMD followed by incubation for various times. Lane C: control. Lane 1: cells incubated for 3 hours after treatment with AMD. Lane 2, 3, 4, 5, 6, 7, and 8: cells incubated for 6, 12, 18, 24, 36, 48 and 72 hours after treatment with AMD.

sis, demonstrated by the increase in the number of both AV single positive and PI single positive cells. Double positive cells (upper right quadrant) represent late apoptotic and necrotic cells. The immunofluorescent detection of DNA breaks by the TUNEL technique (right panel) demonstrates a dose-dependent increase of the number of apoptotic cells.

2. Expression of Bcl-2 and Caspase-3 proteins

Cells were treated with AMD, and the level of Bcl-2 and Caspase-3 proteins expression was detected by western blot analysis. Fig. 4A shows the western blot analysis of the expression of Bcl-2 proteins in K-562 cells treated AMD and then incubated for 3, 6, 9, 12, 18, 24, 36 48 or 72 hours at 37℃. The expression of Bcl-2 proteins was persisted until 3 hours with elevated level, and thereafter was disappeared after 12 hours incubation. When the same experiment was performed with K-562 cells, level of Caspase-3 expression was gradually increased from incubation for 6 hours (Fig. 5B). These results suggest that AMD induced apoptosis of K-562 cells via Caspase-3-dependent fashion, and this apoptosis is related to disappearance of Bcl-2 proteins.

DISCUSSION

The exposure to DNA damaging stress like mutagenic and clastogenic agents is known to induce an apoptosis in eukaryotic cells (Wolff et al., 1991, Ames et al., 1995). Many DNA damaging agents including radiation and anticancer drugs are known to induce apoptosis and some efforts were made to elucidate the mechanism of DNA damaging agent induced apoptosis (Marks and Fox, 1991; Martin et al., 1993). DNA damaging agents produce single or double strand breaks, and morphological changes of apoptosis are associated with double strand breaks of nuclear DNA at the linker regions between nucleosomes. This breaks produces ladders of DNA fragmentation that are the sizes of integer multiples of a nucleosome length (180~200 bp), and these nucleosomal DNA ladders are widly used as biochemical markers of apoptosis (Wyllie et al., 1980; Fesus et al., 1991; Dedera et al., 1993). DNA ladder pattern shown in fish OCP 13 cells at 6 hour after treated with 20 J/m^2 UV (Nishigaki et al., 1999), and in human monoblastoid U937 cells treated with 5 μg/ml actinomycin D for 1 hour (Samali and Cotter, 1996). And DNA ladder pattern was evident when the HL-60 (human leukemia) cells were heated at 42℃ for 1 hour, and then incubated at 37℃ for 3 hours (Takasu et al., 1998). In the present study, DNA fragmentation induced by treatment with 10 μg/ml AMD was shown at 6 hour. These results are generally consistent with others' reports. Thus, the inidence of apoptosis is variable depending on the cell type or DNA damaging agents. This may imply that the machineries involved in DNA repair and other sensing machineries to external apoptotic stimuli are variable in different cell type and also with other apoptosis-inducing agents.

In an effort to elucidate mechanisms underlying the different incidences of apoptosis in K-562 cells, expression levels of Bcl-2 and Caspase 3 proteins, were measured. The Bcl-2 proteins, originally described in lymphoma cells (Tsujimoto et al., 1987) and then found to be widely distributed in a variety of cancerous tissues (Reed JC et al., 1995) (Adams JM et al., 2001), is a potent inhibitor of cell death, both programmed and accidental (Kane DJ et al., 1993) (Kroemer G et al., 1998). Overexpression of Bcl-2 and Bcl-XL inhibits apoptosis induced by a variety of stimuli, in particular those that utilize the mitochondria-dependent pathway (Gross A et al., 1999). Overexpression of Bcl-2 is known to convey resistance to apoptosis induced by many agents (Adams JM et al., 1998). In human promyeloid leukemia cells and B-cell lymphoma cells, Bcl-2 overexpression results in up-regulation of capacitative Ca^{2+} entry and resistance to apoptosis induced by the inhibitor of capacitative Ca^{2+} entry. By Nancy et al., was demonstrated acute Bcl-2 overexpression by transient transfection

can induce apoptosis in HEK 293 and MDA-MB-468 cells (Wang NS et al, 2001). In this study, we found that the expression of Bcl-2 proteins was persisted until 3 hours with elevated level, and thereafter was disappeared after 12 hours incubation. Considering, previous reports by other and the present data, the Bcl-2 proteins, when overexpressed, are able to block DNA fragmentation associated with apoptosis.

Mamalian caspases are a family of cysteine proteases that plays a critical role in apoptosis (Thornberry NA et al., 1998). Caspase is known to be essential for apoptosis in a variety of species and model. Because Caspase activities can be detected in all cells undergoing apoptosis, regardless of their origin or death stimuli, and they bring about most of visible changes that characterize apoptotic cell death, Caspases can be thought as the central executioners of apoptosis. During apoptosis, activation of Caspases is a common feature that leads to the cleavage of specific substrates including many signaling proteins (Thornberry NA et al., 1998). Caspase-3 also plays a central role in the execution of the apoptotic program. Caspase-3 is one of the earliest identified caspase, but the mechanism of Caspase-3 induced apoptosis remains unknown. Considering, previous reports by other and the present data, the increment of Caspase-3 proteins can activaties DNA fragmentation associated with apoptosis in K-562 cells. These results suggest that AMD induced apoptosis of K-562 cells via Caspase-3-dependent fashion, and this apoptosis is related to disappearance of Bcl-2 proteins. To elucidate the detailed molecular mechanisms of DNA fragmentation associated with apoptosis further studies are necessary.

REFERENCES

Adams JM, Cory S. Life-or-death decisions by the Bcl-2 protein family. Trends Biochem Sci. 2001. 26: 61-66.

Adams JM, Cory S. The Bcl-2 protein family: Arbiters of cell survival. Science 1998. 281: 1322-1326.

Ames BN, Gold LS, Willett WC. The Causes and Prevention of Cancer. Proc Natl Acad Sci USA. 1995. 92: 5258-5265.

Arends MJ, Morris RG, Wyllie AH. Apoptosis: the role of the endonuclease. Am J Pathol. 1990. 136: 593-608.

Barry MA, Behnke CA, Eastman A. Activation of programmed cell death (apoptosis) by cisplastin, other anticancer drugs, toxins and hyperthermia. Biochem Pharmacol. 1990. 40: 2353-2362.

Batistatou A, Greene L. Internucleosomal DNA cleavage and neuronal cell survival/death. J Cell Biol. 1993. 122: 523-532.

Cohen JJ, Duke RC. Apoptosis and programmed cell death in immunity. Annu Rev Immunol. 1992. 10: 267-293.

Dedera DA, Waller EK, LeBrun DP, Sen-Majumdar A, Stevens ME, Barsh GS, Cleary ML. Chimeric homeobox gene E2A-PBX1 induces proliferation, apoptosis, and malignant lymphomas in transgenic mice. Cell 1993. 74: 833-843.

Farooqi Z, Kesavan PC. Low-dose radiation-induced adaptive response in bone marrow cells of mice. Matat Res. 1993. 302: 83-89.

Fesus L, David PJA, Piacentini M. Apoptosis: molecular mechanisms in programmed cell death. Eur J Cell Biol. 1991. 56: 170-177.

Gross A, McDonnell JM, Korsmeyer SJ. Bcl-2 family members and the mitochondria in apoptosis, Genes Dev. 1999. 13: 1899-1911.

Jones DP, McConkey DJ, Nicotera P, Orrenius, S. Calcium-activated DNA fragmentation in rat liver nuclei. J Biol Chem. 1989. 264: 6398-6403.

Kane DJ, Sarafian TA, Anton R, Hahn H, Gralla EB, Valentine JS, Ord T, Bredesen DE. Bcl-2 inhibition of neural death: decreased generation of reactive oxygen species, Science 1993. 262: 1274-1277.

Kroemer G, Dallaporta B, Resche-Rigon M. The mitochondrial death/life regulator in apoptosis and necrosis. Annu Rev Physiol. 1998. 60: 619-642.

Lennon SV, Martin SJ, Cotter TG. Dose-dependent induction of apoptosis in human tumor cell lines by widely diverging stimuli. Cell Prolif. 1991. 24: 203-214.

Liu XH, Collier RJ, Youle RJ. Inhibition of axotomy-induced neuronal apoptosis by extracellular delivery of a Bcl-XL fusion protein. J Biol Chem. 2001. 276: 46326-46332.

Martin DS, Stolfi RL, Colofiore JR, Koutcher JA, Alfieri A, Sternberg S, Nord, LD. Apoptosis regulating from anti-cancer agent activity in vivo is enhenced by biochemical modulation of tumor cell energy. Programmed cell death: the cellular and molecular biology of apoptosis.(Lavin, M. and Watters, D., ed) Harwood academic publishers, Postrasse, Switzerland. 1993. 279-296.

Marks DI, Fox RM. DNA damage, poly(ADP-ribosyl)ation and apoptotic cell death as a potential common pathway of cytotoxic drug action. Biochem Pharmacol. 1991. 42: 1859-1867.

McConkey DJ, Hartzell P, Jondal M, Orrenius S. Inhibition of

DNA fragmentation in thymocytes and isolated thymocyte nuclei by agent that stimulate protein kinase C. J Biol Chem. 1989. 264: 13399-13402.

Nagano M, Ui-Tei K, Suzuki H, Piao Z, Miyata Y. CDK inhibitors suppress apoptosis induced by chemicals and by excessive expression of a cell death gene, reaper, in *Drosophila* cells Apoptosis 2000. 5: 543-550.

Nishigaki R, Mitani H, Tsuchida N, Shima A. Effect of cyclobutane pyrimidine dimers on apoptosis induced by different wavelengths of UV. Photochem Photobiol. 1999. 70: 228 -235.

Reed JC. Regulation of apoptosis by Bcl-2 family proteins and its role in cancer and chemoresistance. Curr Opin Oncol. 1995. 7: 541-546.

Rudner J, Jendrossek V, Belka C. New insights in the role of Bcl-2 and the endoplasmic reticulum. Apoptosis 2002. 27: 441 -447.

Samali A, Cotter TG. Heat shock proteins increase resistance to apoptosis. Exp Cell Res. 1996. 223: 163-170.

Soldani C, Lazze MC, Bottone MG, Tognon G, Biggiogera M, Pellicciari CE, Scovassi AI. Poly (ADP-ribose) polymerase cleavage during apoptosis: when and where? Exp Cell Res. 2001. 269: 193-201.

Takasu T, Lyons JC, Park HJ, Song CW. Apoptosis and perturbation of cell cycle progression in an acidic environment after hyperthermia. Cancer Res. 1998. 58: 2504-2508.

Towbin H, Staehelin T, Gordon J. Electrophoretic transfer of proteins from acrylamide gels to nitrocellulose sheet: Procedure and some applications. Proc Natl Acad Sci USA. 1979. 76: 4350-4358.

Tsujimoto Y, Ikegaki, N, CroceC. M. Characterization of the protein product of Bcl-2, the gene involved in human follicular lymphoma. Oncogene 1987. 2: 3-7.

Thornberry NA, Lazebnik YN. Caspases: Eneemics Within. Science 1998 281: 1312-1316.

Vermes I, Haanen C, Steffens-Nakken H. A novel assay for apoptosis. Flow cytometric detection of phosphatidylserine expression on early apoptotic cells using fluorescein labelled annexin V. J Immunol Methods. 2001. 184: 39-51.

Wang NS, Unkila E, Reineks Z, Distelhorst CW. Transient expression of wild-type or mitochondrially targeted Bcl-2 induces apoptosis, whereas transient expression of endoplasmic reticulum-targeted Bcl-2 Is protective against Bax-induced cell death. J Biol Chem. 2001. 276: 44117-44128.

Waring P. DNA fragmentation induced in macrophage by gliotoxin does not require protein synthesis and is preceded by raised inositol triphosphate levels. J Biol Chem. 1990. 265: 14476-14480.

Wolff S, Jostes R, Cross FT, Hui TE, Afzal V, Wiencke JK. Adaptive response of human lymphocytes for the repair of radon-induced chromosomal damage. Mutat Res. 1991. 250: 299 -306.

Wyllie AH, Kerr JFR, Currie AR. Cell death: the significance of apoptosis. Int Rev Cytol. 1980. 68: 251-306.

Wyllie AH, Morris RG, Smith AL, Dunlop D. Chromatin cleavage in apoptosis: association with condensed chromatin morphology and dependence on macromolecular synthesis. J Pathol. 1984. 142: 67-77.

Yuan J. Evolutionary conservation of a genetic pathway of programmed cell death. J Cell Biochem. 1996. 60: 4-11.