CISPLATIN CYTOTOXICITY IS UNALTERED BY BCL-2 EXPRESSION

BY

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B.Sc. BIOCHEMISTRY

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ABSTRACT

A major challenge in cancer therapy is the emergence of acquired resistance to a wide range of chemotherapeutic drugs with unrelated structures and activities. Possible mechanisms to explain drug resistance are induction of efflux pumps, activation of scavenging pathways and/or changes in the oncogene status of a cell. A number of studies have shown that overexpression of Bcl-2 confers resistance by preventing drug induced apoptosis. In this thesis, Madin Darby canine kidney epithelial (MDCK) cells were used to investigate the relationship between cisplatin resistance and Bcl-2 expression. In our studies overexpression of Bcl-2 was sufficient in preventing apoptosis induced by serum deprivation. However, treatment with varying cisplatin doses did not induce an apoptotic response. Electron microscopy and in situ DNA end labelling experiments show changes distinct from those associated with serum deprivation-induced apoptosis. Survival as assessed by DNA fluorometry and clonogenic assays clearly demonstrate that the overexpression of Bcl-2 fails to protect against the cytotoxic effects of cisplatin in MDCK cells. Our results show that cisplatin induces a form of cell death distinct from apoptosis and suggests that multiple pathways to cell death exist which are differentially regulated in a cell type-specific and stimuli-specific fashion.

DEDICATIONS

This thesis is dedicated to my family whose unconditional love and encouragement has always inspired me through even the most difficult times.

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CHAPTER ONE

INTRODUCTION

Cancer has been examined with the viewpoint that cells have acquired the ability to proliferate in an unregulated fashion. Consequently, the aim of therapy has been to limit the proliferation of cancerous cells. Over the past two decades, the study of normal and tumor cell biology in terms of examining the role of programmed cell death has become important. The notion that the spontaneous and continuous loss of cells is an important parameter of malignant neoplasms was recognized in the late 1960s (1,2). In 1972, the role of programmed cell death as it relates to both healthy and neoplastic tissues was examined. This unique form of cell death became known as "apoptosis"; a Greek word describing the "falling off" of leaves from a tree. Apoptosis provides an efficient mechanism for eliminating unwanted cells and is therefore often referred to as "self-directed

cellular suicide". Apoptosis was originally defined morphologically and attempts were made to distinguish this programmed cell death from the pathological cell death known as necrosis (3).

Necrosis is most often observed when cells die from severe and sudden injury and is referred to as "accidental cell death". Necrosis can be detected morphologically by signs of irreversible injury which include cell swelling, random chromatin clumping into ill-defined masses of non specific DNA cleavage and gross swelling of organelles. At a later stage, the plasma membrane breaks down leading to disruption of the cell with the release of its cytoplasmic contents into the extracellular fluid. In contrast, early morphological changes associated with apoptosis include: changes in cell shape which may involve cytoskeletal alterations, chromatin aggregation into large masses on the nuclear membrane and the nuclear outline becomes grossly indented. Concomitant with these nuclear changes, the cytoplasm begins to condense, microvilli disappear and blunt protuberances are formed on the cell surface. At a later stage, cleavage of DNA into specific oligonucleosome sized fragments is

observed which produces a DNA ladder, the nucleus is broken down into discrete fragments with condensed chromatin and eventually the cell separates itself into membrane bound apoptotic bodies which can be taken up by neighbouring phagocytic cells (4,5).

APOPTOSIS AND CANCER CHEMOTHERAPY

Many chemotherapeutic drugs do not afford a selective means of preventing tumor growth without affecting that of normal tissues and some of the major cancers continue to remain largely resistant to such therapy. The cytotoxicity of a drug is based largely on the interactions with its target. Unfortunately, selecting one particular event as a potential drug target, from the multitude of events likely to occur prior to the development of a malignant cell is challenging. More recently, it has been suggested that the initial drug-target stimulus must somehow be coupled to a molecular response within the cell, the most desired response being programmed cell death. A model was proposed suggesting that the initial drug-target interaction and the events that follow function as a signal in the induction of apoptosis.

Therefore, apoptosis may represent a conserved adaptative response to cellular damage (6-8). To date, numerous anticancer agents with disparate modes of action have been found to elicit an apoptotic response (Table 1).

TABLE 1: A BRIEF LIST OF CYTOTOXIC AGENTS CAPABLE OF INDUCING APOPTOSIS

AGENT	CELL TYPE	RESPONSE	REF
Glucocorticoids	immature rat thymocytes	typical[1]	(9)
etoposide	immature rat thymocytes	typical	(10)
	myelocytic leukemia	typical	(10)
camptothecin	immature rat thymocytes	atypical[2]	(11)
	human promyelocytic leukemia	typical	(12)
cisplatin	murine IL-3 dependent	typical	(13)
	human ovarian carcinoma	atypical	(14)
	human promyelocytic leukemia	typical	(12)
	chinese hamster ovary	typical	(15)
	rat hepatoma	typical	(16)
nitrogen mustard	Burkitts lymphoma	typical	(17)
	murine IL-3 dependent	typical	(18)
	human promyelocytic leukemia	typical	(12)
5-fluorouracil	mouse FM3A	typical	(19)
Ara-C	human myeloid leukemia	typical	(20)
radiation	rat 208F fibroblast	typical	(21)
	mouse T cell hybridoma	typical	(22)
	human promyelocytic leukemia	typical	(12)
PDT	human prostate	typical	(23)
	rat mammary carcinoma	typical	(23)

1- "typical" refers to the presence of endonucleolytic cleavage DNA pattern
2- "atypical" refers to cleavage of DNA into larger 50-300kb fragments
The abbreviations used are: Ara-C: 1-β-D-arabinofuranosyl-cytosine; PDT: photodynamic therapy

The ability of cancer cells to induce apoptosis became an important factor in optimizing therapeutic response. However, the mechanisms which function in the pathway linking drug induced damage to apoptosis remain largely unknown. It has been proposed that the induction of apoptosis in response to drug treatment may vary according to both the cell type and the damaging agent (8,15).

Cis-diamminedichloroplatin(II) (cisplatin, DDP) is commonly used in the treatment of testicular, bladder, head and neck, cervical cancer and is the primary therapy for ovarian cancer (24). DNA is believed to be the critical target for cisplatin. The ability of cisplatin to interact with DNA is the result of its electrophilic nature. Lower chloride ion concentrations within a cell facilitate the exchange of chloride ions on cisplatin for hydroxyl or water groups. Most of the DNA damage that is produced is due to the formation of platinum-DNA intrastrand adducts and less frequently to interstrand and DNA-protein adducts (25). Cytotoxicity associated with cisplatin was originally believed to be the result of the inhibition of DNA synthesis. It was later shown that DNA repair-deficient chinese hamster ovary (CHO) cells died at

cisplatin concentrations that did not inhibit DNA synthesis (26). The finding that inhibition of DNA synthesis is not solely responsible for toxicity challenged investigators to determine a link between DNA adduct formation and induction of cell death. In CHO cells, cisplatin induced transient G_2 arrest at low cisplatin concentrations, but at higher concentrations G_2 arrest was accompanied by the formation of a DNA ladder (27). These observations suggested the involvement of an endogenous endonuclease and it was concluded that cisplatin induces apoptosis (28). Since these observations it was determined that cisplatin induces apoptosis in many cell types (12,15,16,29-31).

The initial success with cisplatin clinically is often limited with the development of a resistant tumor cell population (32,33). Little is known about the exact mechanism(s) of resistance to platinating agents. Some proposed mechanisms of resistance include:i) decreased intracellular accumulation or decreased influx, ii) decreased intracellular binding/sequestration and/or increased efflux, iii) enhanced detoxification by scavenger molecules such as glutathione and/or metallathioneins and iv) increased DNA repair (34). The difficulty in

investigating cisplatin resistance is that multiple factors appear to contribute to resistance in a given population and such factors are highly variable between cell populations. With the knowledge that cisplatin induces apoptosis, emergence of cisplatin resistance as a consequence of evasion of the apoptotic pathway
is of current interest.

GENETIC CONTROLS ON APOPTOSIS: THE ROLE OF BCL-2

Most of our knowledge on oncogenic events focuses on mechanisms involved in proliferation and cell growth. Two classes of proto-oncogenes appear to be important in this area: a) genes involved in the control of transcription and signal transduction (c-myc, ras, abl) and b) tumor suppressor genes (p53, rb) in which a loss of function mutation results in unchecked growth. The proto-oncogene bcl-2 however, is unique because it has been found to inhibit apoptosis, prolonging cell survival without cell proliferation.

The bcl-2 (B-cell leukemia/lymphoma 2) gene was first isolated from the breakpoint of the translocation between chromosome 14 and

18 in follicular B-cell lymphomas (35-37). The direct involvement of the bcl-2 gene in follicular lymphoma came from evidence demonstrating that bcl-2 gene expression is activated by the t(14;18) translocation (38). This translocation retains the protein coding region of the bcl-2 gene intact and structurally unaltered but deregulates its expression due to the proximity of the immunoglobulin heavy chain enhancer elements (39-41). Cells which possess the t(14;18) translocation have correspondingly elevated Bcl-2 protein levels (42).

The Bcl-2 protein lacks any obvious internal signal motifs but has been found to associate with membranes via a carboxy-terminal hydrophobic domain (43). Biochemical fractionation, electron and confocal microscopy have demonstrated that Bcl-2 is present in the inner and outer mitochondrial membrane (43,44), the plasma membrane (45), the endoplasmic reticulum membrane (42) and the nuclear membrane (44). However, the site at which Bcl-2 functions in preventing apoptosis remains to be determined.

The ability of Bcl-2 to prolong cell survival has been demonstrated in IL-3 dependent murine haemopoietic cells. These cells die by apoptosis in the absence of the cytokine (46) but IL-3 dependent cells overexpressing Bcl-2 are prevented from entering apoptosis (47). This study demonstrates that Bcl-2 can suppress apoptosis and prolong the survival of these cells in an environment in which they would otherwise die. Interestingly, other cell lines requiring IL-2 or IL-6 did not survive longer following cytokine removal when Bcl-2 was overexpressed (48). It appears that protection by Bcl-2 may be associated with particular cell lineages and cell conditions.

A transgenic mouse model developed using minigene constructs that mimic the consequence of the t(14;18) chromosome translocation was generated to study the effects of bcl-2 gene deregulation in vivo (49,50). Enhanced B cell survival was observed in the lymphoid organs of mice bearing the bcl-2-Ig transgene and following a period of 15 months, these mice developed B-cell lymphomas. This transgenic mouse model demonstrates that Bcl-2 prolonged B-cell

survival and other genetic events likely complemented Bcl-2 expression leading to transformation and tumor progression.

While Bcl-2 plays a major role in the pathogenesis of B cell lymphomas, it has also been shown that Bcl-2 confers resistance to a wide spectrum of chemotherapeutic drugs (Table 2). Although some drug resistance in cancer cells is due to the overexpression of the multiple drug resistance gene (MDR), not all drug resistance can be explained by MDR expression. The overexpression of Bcl-2 has presented an attractive alternative in achieving multidrug resistance. Bcl-2 has been experimentally shown to render cells more resistant to apoptosis induced by dexamethasone, cytosine arabinoside (Ara-C), methotrexate, 5-fluorodeoxyuridine, etoposide (VP-16), vincristine, cisplatin, nitrogen mustard and camptothecin (13,18,51,52).

It has been noted that while Bcl-2 prevents many forms of apoptosis in a wide range of cell systems, it does not prevent all forms of apoptotic cell death (53). These observations suggest that multiple pathways to apoptosis may exist, some which are Bcl-2 dependent and others which are unaffected by Bcl-2 gene expression. A family

of Bcl-2 related proteins is emerging which may explain how pathways to apoptosis are differentially regulated.

*TABLE 2: CHEMOTHERAPEUTIC AGENTS TO WHICH BCL-2 CONFERS RESISTANCE

AGENT	CELL LINE	CELL TYPE	REF
methotrexate	697	human pre B cell leukemia	(51)
Ara-C	"	"	(51)
etoposide	"	"	(51)
vincristine	"	"	(51)
cisplatin	"	"	(51)
cisplatin	PC3	human prostate	(54)
FUdR	MUTU-BL	B cell (Burkitts lymphoma)	(52)
methotrexate	S49.1/WEH17.2	thymus derived T cells	(13)
vincristine	"	"	(13)
cisplatin	SHEP-1	human neuroblastoma	(55)
etoposide	"	"	(55)
etoposide	PC3	human prostate	(54)}
camptothecin	FL5.12	murine IL-3 dependent	(18)
HN2	FL5.12	"	(18)

* Table adapted from (56).
The abbreviations used are: Ara-C: 1-β-D-arabinofuranosyl-cytosine, FUdR: 5-Fluorodeoxyuridine, HN2: nitrogen mustard.
Cell lines listed express high levels of Bcl-2 and are resistant to the agents indicated as compared to empty vector negative controls.

The findings presented in Table 2 provide evidence that Bcl-2 functions downstream of the initial drug-target interaction by interfering with the apoptotic pathway which may be activated by multiple chemotherapeutic agents. With respect to the DNA damaging agent camptothecin (topoisomerase I inhibitor) quantitation of the number of DNA strand breaks revealed no difference between Bcl-2 transfectants and control cells, even though Bcl-2 protected against camptothecin induced apoptosis (18). The expression of Bcl-2 can inhibit cell death induced by a variety of chemotherapeutic drugs with varying modes of action and even surpasses the list of drugs to which the multidrug resistant phenotype applies. The results of studies using a variety of cell lines must be interpreted with caution since it appears that the ability of Bcl-2 to inhibit apoptosis may be both cell type and agent type specific.

A 21kD protein, Bax, with extensive amino acid homology to Bcl-2 in two highly conserved domains (BH1 and BH2) has been identified (57). The model of Bcl-2 and Bax regulation of apoptosis proposes that the inherent ratio of Bcl-2:Bax determines cell susceptibility to apoptosis following an apoptotic stimulus. Competition between Bcl-2 and Bax via heterodimerization regulates the amount of Bcl-2 or Bax present, such that when Bcl-2 is in excess, cells are protected from apoptosis.

The interaction between Bcl-2 and Bax and the affect on apoptosis, was investigated using a IL-3 dependent murine cell line (FL5.12) transfected with Bax. The induction of apoptosis following IL-3 deprivation was expressed as percent viability. Clones with high Bcl-2:Bax ratios, were viable for over two weeks following IL-3 deprivation. In contrast, clones with low Bcl-2:Bax ratios lost all viability by 7 days. Therefore, it appears that the overexpression of Bax counters Bcl-2 function. In addition to Bax, bcl-x, a bcl-2 related gene has been isolated and shown to function independently of Bcl-2 in regulating apoptosis (58). The greatest similarity between Bcl-x and

Bcl-2 is within a short region at the amino terminus of Bcl-x (which corresponds to a highly conserved domain located internally in Bcl-2) and a hydrophobic domain that appears to mediate membrane integration of the Bcl-2 protein. Two distinct bcl-x mRNA species have been isolated from human tissue, bcl-x_L and bcl-x_S. The bcl-x_L gene encodes a protein that is similar in size and structure to Bcl-2. The shorter, bcl-x_S gene encodes a 170 amino acid protein in which the region of highest homology between Bcl-x_L and Bcl-2 has been deleted (58).

Stable Bcl-x_L FL5.12 transfectants were significantly resistant to apoptosis compared to control transfected counterparts and the resistance conferred was greater than that by Bcl-2 alone. In addition, co-transfection of Bcl-x_L and Bcl-2 did not improve cell survival beyond that observed with Bcl-x_L alone. In contrast, Bcl-x_S had no effect on the rate of apoptosis and when co-transfected with Bcl-2, FL5.12 cells acquired sensitivity to IL3 withdrawal and underwent apoptosis. These observations suggest that Bcl-x_S can function as a negative regulator of Bcl-2 (58).

Interactions among members of the Bcl-2 protein family were investigated using the yeast two hybrid system (59,60). Based on the results obtained the following model was proposed: i) heterodimerization of Bax with Bcl-2 or Bcl-x_L abrogates Bax function and inhibits apoptosis, and ii) Bcl-x_S selectively interacts with Bcl-2 or Bcl-x_L preventing Bcl-2 or Bcl-x_L from binding to and neutralizing Bax.

AIM OF STUDY

The overexpression of Bcl-2 has been previously shown to inhibit apoptosis induced by cisplatin and prolong cell survival in lymphoid cells (51). In this study, Madin Darby canine kidney epithelial (MDCK) cells overexpressing Bcl-2 were used to investigate whether this is a general phenomenon. The MDCK cell line was derived from the kidney of a normal male cocker spaniel and resembles epithelial cells from the distal tubule (61). Apoptosis has been previously reported in this cell line following treatment with protein toxins such as ricin (62), by hypoxia (63), application of the steroid hormone antagonist RU 486

(64), infection by the influenza virus (65) and by treatment with TGF-β (66).

Different assays were utilized in examining the response to cisplatin treatment and serum deprivation in control and Bcl-2 transfected MDCK cells. Overexpression of Bcl-2 prevented apoptosis caused by serum deprivation, however no difference between control and Bcl-2 transfected MDCK cells in the cell death induced by cisplatin was observed. It was possible to assess survival by DNA fluorometry and colony formation following treatment with a wide range of cisplatin doses and exposure times and it was found that Bcl-2 did not alter cell survival.

The evidence provided in this thesis demonstrates that cisplatin does not induce apoptosis in the MDCK cell model as measured by the assays employed. The data also suggests that multiple pathways to cell death exist which are triggered by different stimuli and regulated differentially in a cell type-specific fashion.

CHAPTER TWO

MATERIALS AND METHODS

2.0 - MATERIALS

Madin Darby canine kidney (MDCK) cells transfected with Bcl-2 were generously provided by Dr. David Andrews and Dr. Brian Leber, McMaster University, Hamilton, Ontario, Canada. Briefly, MDCK cells were stably transfected by calcium phosphate precipitation with a pRC-CMV plasmid encoding full length wild type human Bcl-2 or a pRC-CMV as a control. Preparation of the wild type Bcl-2 clone has been previously described (67). MDCK cells were cultured in α-minimal essential media (MEM) supplemented with 10% fetal bovine serum (FBS) and 400μg/mL G418 (geneticin sulfate) purchased from Gibco, Burlington.

Cisplatin was purchased from Sigma Chemical Co., St. Louis, MO and was prepared as a 500μM stock in sterile PBS, prior to use (140mM NaCl, 2.7mM KCl, 8.1mM Na_2HPO_4, 1.5mM KH_2PO_4, pH 7.4). Cisplatin was diluted in α-MEM to appropriate concentrations. The antibody to Bcl-2 was purchased from Oncogene Science, Uniondale,

NY and was used according to manufacturer's instructions. Prestained molecular weight protein markers were obtained from Sigma Chemical Co., St. Louis, MO.

2.1 - IMMUNOBLOTTING

Protein samples were prepared from approximately 10^7 cells pelleted by centrifugation at 200g (IEC Centra-8R centrifuge R12 Type for 5 minutes and then lysed in 50mM Tris pH8, 150mM NaCl, 1% nonidet P-40, 20μg/ml chymostatin, antipain, leupeptin, pepstatin, 40μg/ml aprotinin and 100μg/ml PMSF. The solubilized cell proteins were then recovered in the supernatant fraction after centrifugation at approximately 10,000g (Eppendorf Centrifuge 5415C) for 10 minutes at 4°C. Protein samples of approximately 10μg were separated electrophoretically by SDS-PAGE and transferred onto a nitrocellulose membrane using the BioRad Mini-Trans Blot apparatus. Transfer was performed at 30V and completed in 2 hours. The gel, nitrocellulose and Whatman 3MM chromotography paper were soaked in TBST buffer (10mM Tris pH 8, 150mM NaCl, 0.05% Tween-20) prior to

assembly of transfer apparatus. The blots were blocked for one hour in 5% skim milk; 0.05% Tween-20; PBS at 4°C. The primary antibody was added (1:100) and incubated at room temperature for 1 hour. The nitrocellulose membrane was washed twince in TBST for 10 minutes each wash and then incubated with a secondary antibody(goat anti-mouse IgG conjugated to horseradish peroxidase, Oncogene Science, Uniondale, NY) for 1/2 hour and developed using enhanced chemilumenescence (Amersham).

2.2 - ASSAY OF GENOMIC INTEGRITY BY DNA GEL ELECTROPHORESIS

Approximately 5 x 10^5 cells were seeded onto 100mm Falcon tissue culture dishes and allowed to adhere overnight. On the following day, cells were treated with one of the protocols outlined below.

2.2.1-SERUM STARVATION

Tissue culture plates were rinsed three times with sterile PBS and 10mL of α-MEM media supplemented with 0.1% FBS was added.

Plates were incubated at 37°C,5% CO_2 in a humidified chamber for 48 hours.

2.2.2-CISPLATIN TREATMENT

The samples were incubated with appropriate cisplatin concentrations for 2 hours, then replaced with fresh α-MEM media supplemented with 10% FBS and incubated at 37°C, 5% CO_2 in a humidified chamber for the desired post treatment incubation period.

Following the appropriate treatment protocol, both nonadherent and adherent cells were collected. Adherent cells were collected using 1.5mL of 10X trypsin-EDTA and pelleted by centrifugation for 5 minutes in a IEC Centra-8R centrifuge. The resulting cell pellets were resuspended in 400μL of Hirt Lysis buffer (0.6% SDS, 10mM EDTA pH 8, 10mM Tris pH 8) producing a gelatinous mass which was transferred to an eppendorf containing 200μL of cold 3M sodium acetate, pH 8.0. High molecular weight DNA was removed by centrifugation at 10,000g for 30 minutes in a Eppendorf 5415C microcentrifuge. Low molecular weight DNA was isolated from the supernatant by standard phenol chloroform extraction. DNA was

precipitated in 2.5X volume of cold 95% ethanol and stored overnight at -20°C . On the following day, low molecular weight DNA was pelleted by centrifugation at 10,000g for 15-20 minutes (at 4°C). The resulting pellets were then resuspended in 50μl of 1X TE buffer (10mM Tris pH 7.4, 10mM EDTA pH 8) with 20μg/ml boiled RNAse A and incubated at room temperature for 10 minutes. Electrophoresis was carried out in a 1.5% agarose gel containing ethidium bromide in a final concentration of 0.5μg/ml, similar concentrations of ethidium bromide were used in the running buffer, 25μl of sample with 5μl of gel loading buffer (0.25% bromophenol blue, 0.25% xylene cyanol FF, 30% glycerol in water) was loaded onto the gel and DNA was separated electrophoretically for 2 hours at 75V. DNA was visualized and photographed under UV transillumination.

2.3-IDENTIFICATION OF CELL DEATH IN SITU

Apoptosis was also assessed in situ using the Apotag technique purchased from Oncor and was carried out according to manufacturer's instructions. Briefly, cells were treated and grown on

coverslips followed by fixation in 1.0% paraformaldehyde for 5 minutes. Endogenous peroxidase was inactivated by immersing coverslips in a 2.0% hydrogen peroxide solution for 5 minutes. The coverslips were rinsed in PBS (50mM sodium phosphate, pH 7.4, 200mM NaCl) and incubated for 1 hour at 37°C in a buffer containing digoxigenin-11-dUTP (DIG-dUTP) and terminal deoxynucleotidyl transferase (TdT). The incorporation of labelled nucleotides was detected by peroxidase conjugated anti-digoxigenin antibodies and was visualized by a colour reaction produced with diaminobenzidine. The coverslips were then counterstained in 1% (w/v) methyl green in 0.1M sodium acetate, pH 4 for 10 minutes, followed by a rinse in distilled water and a wash in 100% butanol. Finally, the coverslips were dehydrated with xylene and mounted using permount mounting media. Photographs were taken using a LEICA light microscope.

2.4-ELECTRON MICROSCOPY

Approximately 10^6 cells were seeded onto 100mm Falcon tissue culture dishes and allowed to adhere overnight. On the following day

cells were cultured in 0.1% FBS supplemented media for 48 hours or treated with desired cisplatin concentrations for 2 hours and then cultured in drug free media for 48 hours. Both adherent and nonadherent cells were collected by centrifugation at 600g (IEC Centra-8R Centrifuge) for 5 minutes. The resulting pellet was washed once in 1ml of sterile PBS and then fixed with 100μl of glutaraldehyde(in 0.1M sodium cacodylate, pH 7.4) for no more than 30 minutes. The following procedure was performed by Ernie Spitzer and other members of the Electron Micrscopy Department at McMaster University. Samples were rinsed twice (5 minutes each) in 0.2M sodium cacodylate and post fixed in 1% osmium tetroxide (in 0.1M sodium cacodylate, pH7.4). Dehydration was achieved through a series of ethanol washes followed by propylene oxide treatment. Samples were then infiltrated with Spur's resin and polymerized overnight at 65°C. Thin sections (approximately 70nm thick) were cut using an ultramicrotome (Reichert OMU2) and placed onto Cu/Pd 200 mesh grids. Grids were stained with uranyl acetate (5 minutes) and

lead citrate (2 minutes) and viewed using a transmission electron microscope (JEOL 1200 Ex Biosystem).

2.5-SURVIVAL ASSAYS

2.5.1-DNA FLUOROMETRIC ASSAY

Dose response effects in the presence of cisplatin were assessed using the method described by Rago *et al* (68), in which cell number is calculated based on the determination of cellular DNA content. Seeding density and the amount of distilled water employed in cell lysis were modified to accomodate for the use of 24 well plates. Briefly, 5 x 10^3 cells were seeded onto Falcon 24 well plates and allowed to adhere overnight. On the following day, cells were treated with varying cisplatin doses for 2 hours and then incubated at 37°C. Wells containing media with no cells were included in each experiment as a blank reading. Cisplatin was prepared as a 500μM stock solution in sterile PBS and then diluted in α-MEM media supplemented with 10% FBS to give the desired concentration. Following various post treatment periods, plates were removed and the wells were rinsed

gently with sterile PBS and 500μL of distilled water was added to each well. To induce cell lysis, plates were incubated at 37°C overnight and then removed and stored at -80°C. After overnight freezing, plates were left at room temperature for a minimum of 3 hours. In order to determine DNA content, 500μL of Hoescht 33258 (Calbiochem) in 40μg/mL TNE buffer (10mM Tris, 2M NaCl, 1mM EDTA pH 7.4) was added to each well. The plates were read on a cytofluor 2350 fluorescent plate scanner (Millipore, Mississauga, Ont.) using excitation and emission wavelengths of 360 and 460nm respectively.

2.5.2-COLONY FORMING ASSAY

Cells in log phase growth were seeded overnight onto Corning 6 well plates at a seeding density of approximately 100 cells/well and were treated in triplicate for 2 hours with cisplatin concentrations in the range of 0-10μM. Following a 2 hour incubation, drug containing α-MEM media supplemented with 10% FBS was removed and replaced with drug free α-MEM media. Cells were incubated for 6 and 7 days at 37°C, 5% CO_2 in a humidified chamber. Surviving colonies were

stained with 0.5% methylene blue (70% methanol and 30% distilled water) and colonies of 20 or more cells were counted on a light microscope.

CHAPTER THREE

RESULTS

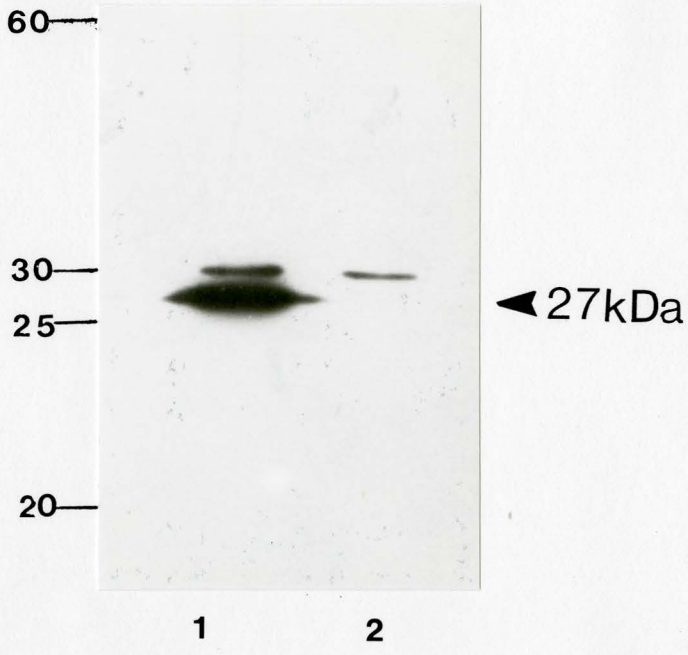
EXPRESSION OF BCL-2 IN MDCK CELLS

To test the hypothesis that Bcl-2 contributes to cisplatin resistance, MDCK cell lines transfected with either a Bcl-2 expression vector or a neo control vector were analyzed for the production of Bcl-2 by immunoblotting using a monoclonal antibody specific to human Bcl-2.

As shown in Figure 1, MDCK(+ Bcl-2) cells (lane 1) express Bcl-2 with a molecular weight of approximately 27 kDa. In contrast, MDCK (- Bcl-2) cells (lane 2) express negligible amounts of Bcl-2. A cross reacting band of unknown identity was also observed.

Figure 1: Immunoblot of cell lysates from MDCK cells transfected with a Bcl-2 expression vector or a vector control

Equal amounts of protein from each sample were loaded onto a 15% SDS polyacrylamide gel. A mouse mAb to human Bcl-2 was used. Lane 1 contains protein from MDCK(+ Bcl-2)cells and lane 2 from MDCK(- Bcl-2) cells.


60
30
25
20
27kDa
1
2

CISPLATIN TREATMENT OF MDCK CELLS

To investigate the mode of cell death, identification of a DNA ladder by conventional gel electrophoresis was chosen as a marker in the detection of cisplatin induced apoptosis. Chinese hamster ovary (CHO) cells treated with cisplatin show DNA fragmentation characteristic of apoptosis (Figure 2). MDCK(- Bcl-2) cells also exhibit DNA ladder fragmentation when cultured in 0.1% serum supplemented media for 48 hours. To determine if MDCK cells show DNA ladder fragmentation following cisplatin treatment similar experiments were performed. MDCK cells were treated with a range of cisplatin doses for 2 hours. Following treatment, cells were cultured in fresh, drug free media for time periods from as early as 8 hours to as late as 48 hours. Untreated controls for both Bcl-2 transfected and control MDCK cells were also included with each cisplatin treated sample. To our surprise, treatment with any of the cisplatin doses used did not induce apoptosis as measured by DNA ladder fragmentation (Figure 3A-J).

Treatment with relatively high cisplatin concentrations and increasing post treatment incubation time to 72 hours still did not result

in DNA ladder fragmentation (Figure 4). Increasing cisplatin concentration did increase the number of cells which detached from the tissue culture plate.

Figure 2: DNA analysis of Chinese Hamster Ovary cells treated with cisplatin

Chinese Hamster Ovary cells were treated with 10μM cisplatin for 2 hours and then cultured in drug free media for 12 hours (lane 1); 24 hours (lane 2) and 48 hours (lane 3). Duplicate samples were run in lanes 4-6. (M) ϕX174 RF DNA/Hae III fragments. Samples were run on a 1.5% agarose gel containing 0.5μg/mL ethidium bromide for 2 hours at 75V. Each lane in the gel represents DNA from an approximately equal number of cells.

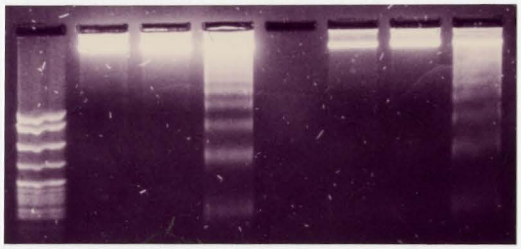

M 1 2 3 4 5 6

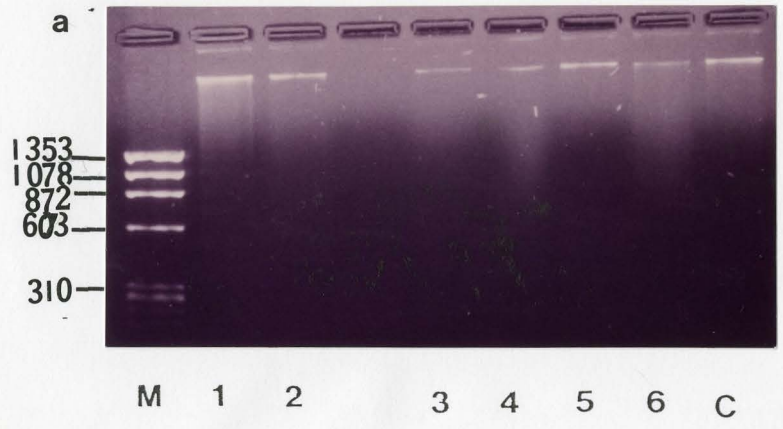
Figure 3: DNA analysis of MDCK cells treated with varying concentrations of cisplatin

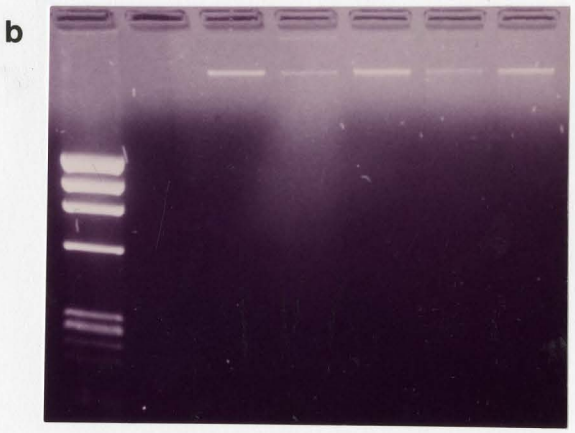
A. Both control and Bcl-2 transfected MDCK cells were treated with **2μM** cisplatin and then cultured in fresh, drug free media. At the appropriate post treatment incubation time DNA samples were prepared and run on a 1.5% agarose gel containing 0.5μg/mL ethidium bromide for 2 hours at 75V.

MDCK(- Bcl-2) cells cultured in drug free media for 8 hours (a, lane 3); 18 hours (a, lane 4); 24 hours (a, lane 5) and 48 hours (a, lane 6). MDCK(+ Bcl-2) cells cultured in drug free media for 8 hours (b, lane 1); 18 hours (b, lane 2); 24 hours (b, lane 3) and 48 hours (b, lane 4). (C) represents untreated MDCK(- Bcl-2) and MDCK(+ Bcl-2) cells cultured for 48 hours. (M) ϕX174 RF DNA/Hae III fragments.

A faint nucleosomal DNA ladder is observed when MDCK(- Bcl-2) cells are serum deprived for 48 hours (a, lane 1). MDCK(+ Bcl-2) cells show no evidence of internucleosomal cleavage when cultured under similar conditions (a, lane 2).

A





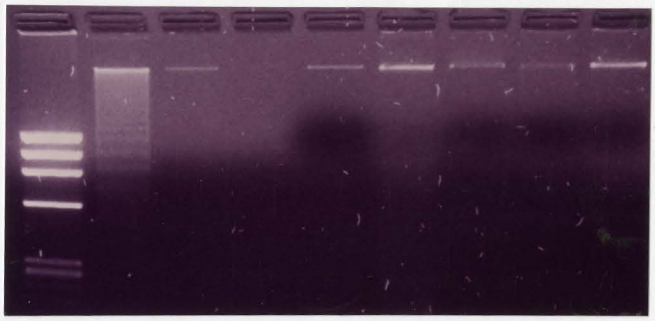
B. Both control and Bcl-2 transfected MDCK cells were treated with **4μM** cisplatin and then cultured in fresh, drug free media. At the appropriate post treatment incubation time DNA samples were prepared and run on a 1.5% agarose gel containing 0.5μg/mL ethidium bromide for 2 hours at 75V.

MDCK(- Bcl-2) cells cultured in drug free media for 8 hours (a, lane 3); 18 hours (a, lane 4); 24 hours (a, lane 5) and 48 hours (a, lane 6). MDCK(+ Bcl-2) cells cultured in drug free media for 8 hours (b, lane 1); 18 hours (b, lane 2); 24 hours (b, lane 3) and 48 hours (b, lane 4). (C) represents untreated MDCK(- Bcl-2) and MDCK(+ Bcl-2) cells cultured for 48 hours. (M) ϕX174 RF DNA/Hae III fragments.

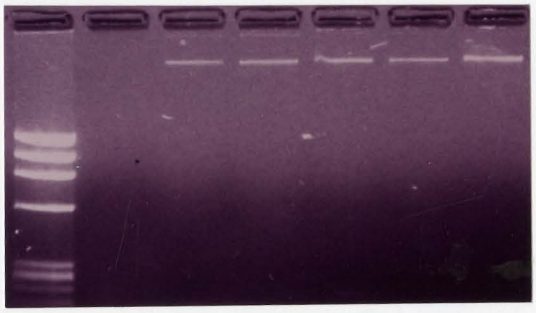
A nucleosomal DNA ladder is observed when MDCK(- Bcl-2) cells are serum deprived for 48 hours (a, lane 1). MDCK(+ Bcl-2) cells show no evidence of internucleosomal cleavage when cultured under similar conditions (a, lane 2).

B

a



b



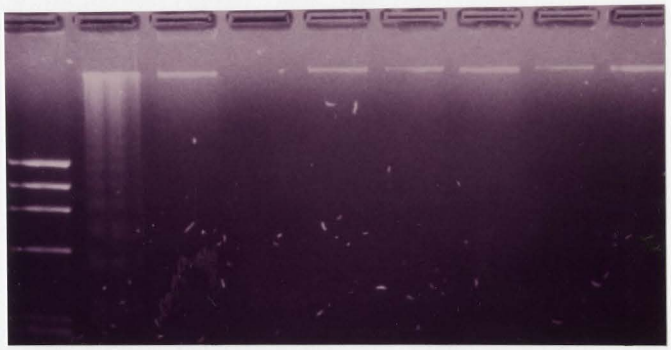
C. Both control and Bcl-2 transfected MDCK cells were treated with **5μM** cisplatin and then cultured in fresh, drug free media. At the appropriate post treatment incubation time DNA samples were prepared and run on a 1.5% agarose gel containing 0.5μg/mL ethidium bromide for 2 hours at 75V.

MDCK(- Bcl-2) cells cultured in drug free media for 8 hours (a, lane 3); 18 hours (a, lane 4); 24 hours (a, lane 5) and 48 hours (a, lane 6). MDCK(+ Bcl-2) cells cultured in drug free media for 8 hours (b, lane 1); 18 hours (b, lane 2); 24 hours (b, lane 3) and 48 hours (b, lane 4). (C) represents untreated MDCK(- Bcl-2) and MDCK(+ Bcl-2) cells cultured for 48 hours. (M) ϕX174 RF DNA/Hae III fragments.

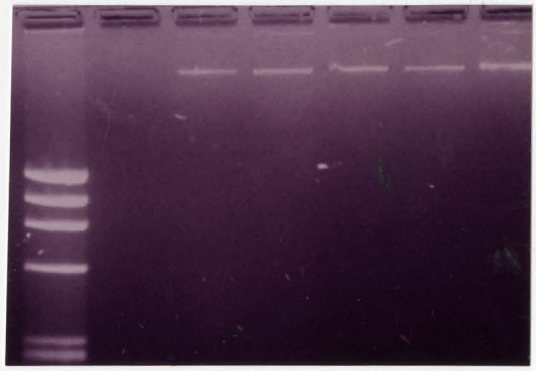
A nucleosomal DNA ladder is observed when MDCK(- Bcl-2) cells are serum deprived for 48 hours (a, lane 1). MDCK(+ Bcl-2) cells show no evidence of internucleosomal cleavage when cultured under similar conditions (a, lane 2).

C

a



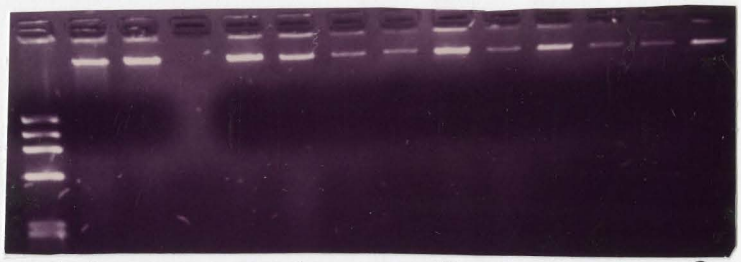
b

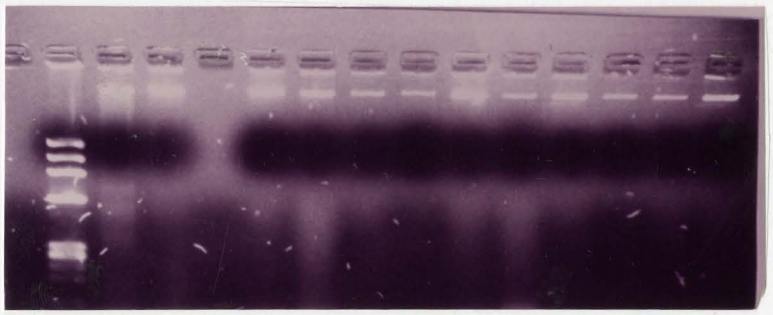


D. Both control and Bcl-2 transfected MDCK cells were treated with **6μM** cisplatin and then cultured in fresh, drug free media. At the appropriate post treatment incubation time DNA samples were prepared and run on a 1.5% agarose gel containing 0.5μg/mL ethidium bromide for 2 hours at 75V.

A nucleosomal DNA ladder is observed when MDCK(- Bcl-2) cells are serum deprived for 48 hours (lane 1). MDCK cells Bcl-2 show no evidence of internucleosomal cleavage when cultured under similar conditions (lane 2). MDCK(- Bcl-2) cells cultured in drug free media for 8 hours (lane 3); 18 hours (lane 4); 24 hours (lane 5) and 48 hours (lane 6). MDCK(+ Bcl-2) cells cultured in drug free media for 8 hours (lane 7); 18 hours (lane 8); 24 hours (lane 9) and 48 hours (lane 10). (C) represents untreated MDCK(- Bcl-2) and MDCK(+ Bcl-2) cells cultured for 48 hours. (M) ϕX174 RF DNA/Hae III fragments. The UV exposure for the bottom gel was increased to 7 seconds.

D

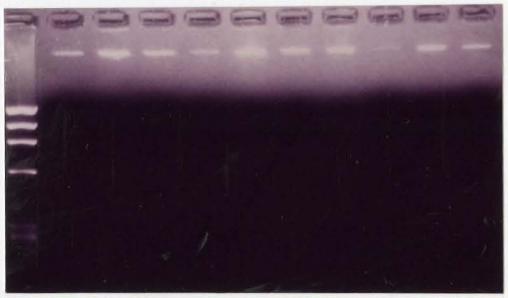




E. Both control and Bcl-2 transfected MDCK cells were treated with **7μM** cisplatin and then cultured in fresh, drug free media. At the appropriate post treatment incubation time DNA samples were prepared and run on a 1.5% agarose gel containing 0.5μg/mL ethidium bromide for 2 hours at 75V.

MDCK(- Bcl-2) cells cultured in drug free media for 8 hours (lane 1); 18 hours (lane 2); 24 hours (lane 3) and 48 hours (lane 4). MDCK(+ Bcl-2) cells cultured in drug free media for 8 hours (lane 5); 18 hours (lane 6); 24 hours (lane 7) and 48 hours (lane 8). (C) represents untreated MDCK(- Bcl-2) and MDCK(+ Bcl-2) cells cultured for 48 hours. (M) ϕX174 RF DNA/Hae III fragments.

E



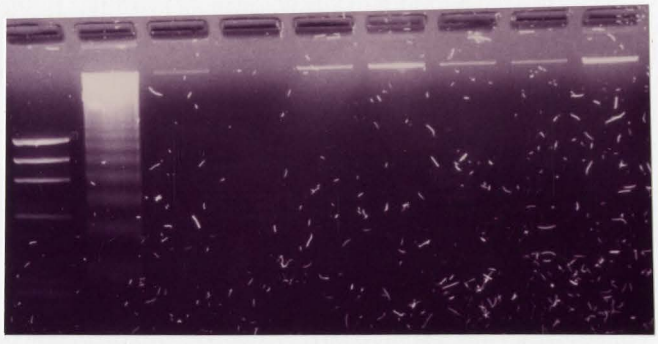
F. Both control and Bcl-2 transfected MDCK cells were treated with **8μM** cisplatin and then cultured in fresh, drug free media. At the appropriate post treatment incubation time DNA samples were prepared and run on a 1.5% agarose gel containing 0.5μg/mL ethidium bromide for 2 hours at 75V.

MDCK(- Bcl-2) cells cultured in drug free media for 8 hours (a, lane 3); 18 hours (a, lane 4); 24 hours (a, lane 5) and 48 hours (a, lane 6). MDCK(+ Bcl-2) cells cultured in drug free media for 8 hours (b, lane 1); 18 hours (b, lane 2); 24 hours (b, lane 3) and 48 hours (b, lane 4). (C) represents untreated MDCK(- Bcl-2) and MDCK(+ Bcl-2) cells cultured for 48 hours. (M) ϕX174 RF DNA/Hae III fragments.

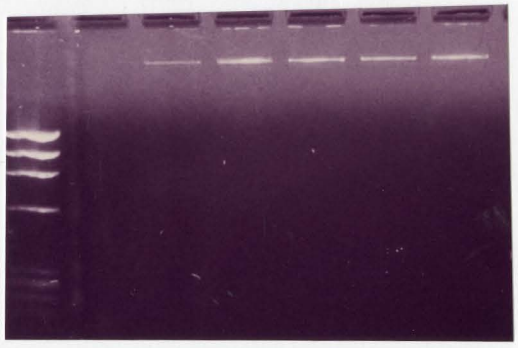
A nucleosomal DNA ladder is observed when MDCK(- Bcl-2) cells are serum deprived for 48 hours (a, lane 1). MDCK(+ Bcl-2) cells show no evidence of internucleosomal cleavage when cultured under similar conditions (a, lane 2).

F

a



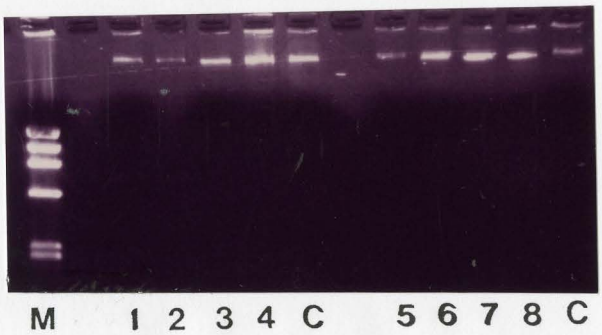
b



G. Both control and Bcl-2 transfected MDCK cells were treated with **9μM** cisplatin and then cultured in fresh, drug free media. At the appropriate post treatment incubation time DNA samples were prepared and run on a 1.5% agarose gel containing 0.5μg/mL ethidium bromide for 2 hours at 75V.

MDCK(- Bcl-2) cells cultured in drug free media for 8 hours (lane 1); 18 hours (lane 2); 24 hours (lane 3) and 48 hours (lane 4). MDCK(+ Bcl-2) cells cultured in drug free media for 8 hours (lane 5); 18 hours (lane 6); 24 hours (lane 7) and 48 hours (lane 8). (C) represents untreated MDCK(- Bcl-2) and MDCK(+ Bcl-2) cells cultured for 48 hours. (M) ϕX174 RF DNA/Hae III fragments.

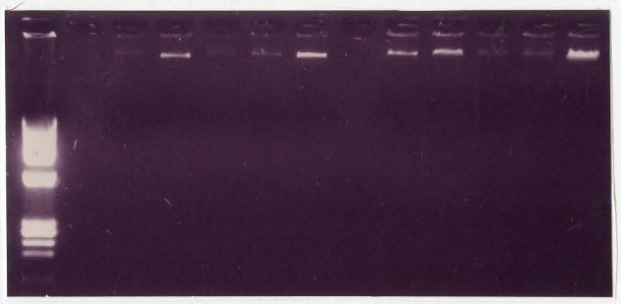
G

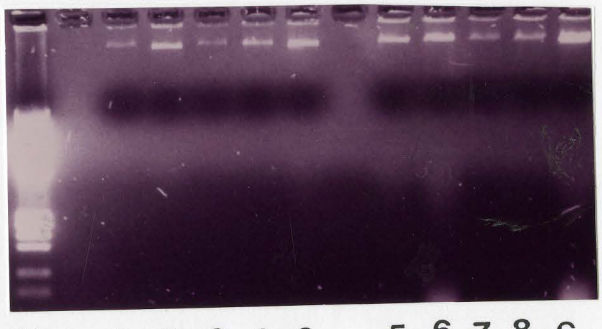


H. Both control and Bcl-2 transfected MDCK cells were treated with **10μM** cisplatin and then cultured in fresh, drug free media. At the appropriate post treatment incubation time DNA samples were prepared and run on a 1.5% agarose gel containing 0.5μg/mL ethidium bromide for 2 hours at 75V.

MDCK(- Bcl-2) cells cultured in drug free media for 8 hours (lane 1); 18 hours (lane 2); 24 hours (lane 3) and 48 hours (lane 4). MDCK(+ Bcl-2) cells cultured in drug free media for 8 hours (lane 5); 18 hours (lane 6); 24 hours (lane 7) and 48 hours (lane 8). (C) represents untreated MDCK(- Bcl-2) and MDCK(+ Bcl-2) cells cultured for 48 hours. (M) ϕX174 RF DNA/Hae III fragments. The UV exposure for the bottom gel was increased to 7 seconds.

H



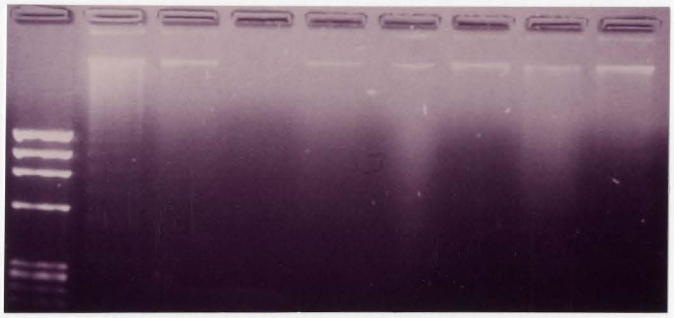


I. Both control and Bcl-2 transfected MDCK cells were treated with **12.5μM** cisplatin and then cultured in fresh, drug free media. At the appropriate post treatment incubation time DNA samples were prepared and run on a 1.5% agarose gel containing 0.5μg/mL ethidium bromide for 2 hours at 75V.

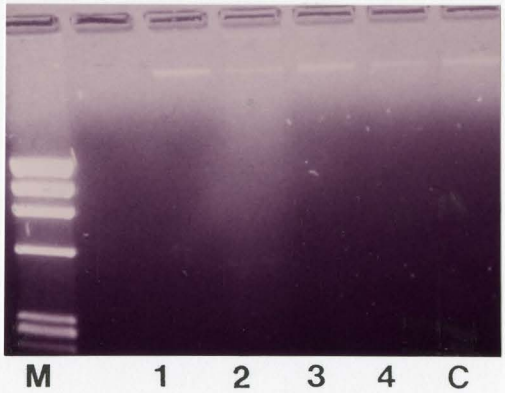
MDCK(- Bcl-2) cells cultured in drug free media for 8 hours (a, lane 3); 18 hours (a, lane 4); 24 hours (a, lane 5) and 48 hours (a, lane 6). MDCK(+ Bcl-2) cells cultured in drug free media for 8 hours (b, lane 1); 18 hours (b, lane 2); 24 hours (b, lane 3) and 48 hours (b, lane 4). (C) represents untreated MDCK(- Bcl-2) and MDCK(+ Bcl-2) cells cultured for 48 hours. (M) ϕX174 RF DNA/Hae III fragments.

A nucleosomal DNA ladder is observed when MDCK(- Bcl-2) cells are serum deprived for 48 hours (a, lane 1). MDCK(+ Bcl-2) cells show no evidence of internucleosomal cleavage when cultured under similar conditions (a, lane 2).

a



b



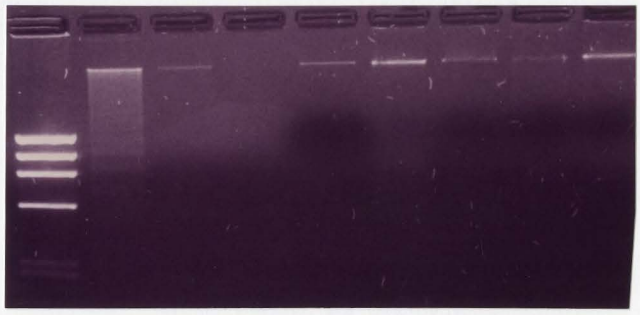
J. Both control and Bcl-2 transfected MDCK cells were treated with **15μM** cisplatin and then cultured in fresh, drug free media. At the appropriate post treatment incubation time DNA samples were prepared and run on a 1.5% agarose gel containing 0.5μg/mL ethidium bromide for 2 hours at 75V.

MDCK(- Bcl-2) cells cultured in drug free media for 8 hours (a, lane 3); 18 hours (a, lane 4); 24 hours (a, lane 5) and 48 hours (a, lane 6). MDCK(+ Bcl-2) cells cultured in drug free media for 8 hours (b, lane 3); 18 hours (b, lane 4); 24 hours (b, lane 5) and 48 hours (b, lane 6). (C) represents untreated MDCK(- Bcl-2) and MDCK(+ Bcl-2) cells cultured for 48 hours. (M) ϕX174 RF DNA/Hae III fragments.

A nucleosomal DNA ladder is observed when MDCK(- Bcl-2) cells are serum deprived for 48 hours (a,b;lane 1). MDCK(+ Bcl-2) cells show no evidence of internucleosomal cleavage when cultured under similar conditions (a,b; lane 2).

J

a



b

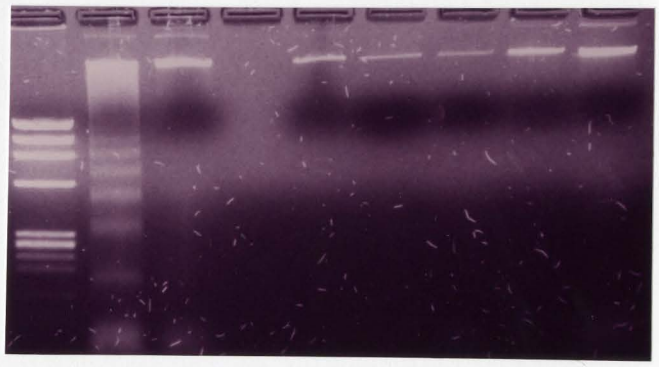
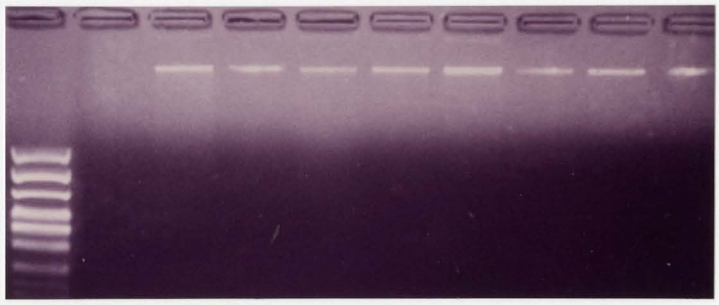


Figure 4: DNA analysis from MDCK cells treated with high cisplatin doses

MDCK cells were treated with 10μM, 12μM and 15μM cisplatin for 2 hours and then cultured in fresh, drug free media for 72 hours. Lanes 1-3 represent DNA from MDCK(- Bcl-2) cells, treated with 10μM (lane 1), 12μM (lane 2) and 15μM (lane 3) cisplatin. Lanes 4-6 represent MDCK(+ Bcl-2) cells treated with 10μM (lane 4), 12μM (lane 5) and 15μM (lane 6) cisplatin. (C) represents DNA from untreated MDCK(- Bcl-2) and MDCK(+ Bcl-2) cells cultured for 48 hours. (M)ϕ-X174/Hae III DNA molecular weight marker.


M
1
2
3
C
4
5
6
C

DETECTION OF CELL DEATH IN SITU

Techniques based on "in situ end-labelling" (ISEL) of DNA strand breaks can be applied to the detection of apoptotic cells (69-73). The DNA strand breaks induced by endonuclease activation in apoptotic cells can be detected using an exogenous enzyme, terminal deoxynucleotidyl transferase (TdT), which incorporates labelled nucleotides in the 3'-hydroxyl termini of DNA fragments. Terminal deoxynucleotidyl transferase catalyzes the template independent addition of deoxynucleotides to the free 3'hydroxyl ends of double or single stranded DNA (74-78).

To detect DNA fragmentation in MDCK cells following either serum deprivation or cisplatin treatment, TdT was used to incorporate digoxigenin labelled dUTP nucleotides in free 3'hydroxyl DNA ends. Enzymatic incorporation was detected indirectly with peroxidase conjugated anti-digoxigenin antibodies. Staining of cells with diaminobenzidine and counterstaining with methyl green facilitated recognition of DNA fragmentation.

Stained cells were recognized as apoptotic if in isolated cells labelled nuclei displayed features of condensation in one or more masses of chromatin. Using these criteria, apoptotic cells were observed in the MDCK(- Bcl-2) cell sample cultured in 0.1% serum supplemented media for 72 hours (Figure 5a, arrowhead). Internucleosomal DNA cleavage in this sample confirmed by gel electrophoresis, correctly identifies these cells as apoptotic (Figure 6). ISEL effectively labelled apoptotic cells following serum deprivation in the MDCK(- Bcl-2) cell sample. In the MDCK(+ Bcl-2) cell sample cultured under identical conditions no evidence of ISEL staining was observed (Figure 5b). Internucleosomal DNA cleavage in this sample confirmed by gel electrophoresis identifies these cells as apoptotic (Figure 6). As expected, no ISEL staining was observed when the ISEL reaction was performed without TdT in the labelling mix (Figure 7a,b) and when MDCK cells were grown in complete supplemented media for 48 hours (Figure 8c,d respectively). Peroxidase staining was also observed in MDCK cells following cisplatin treatment (Figure 8), producing a staining pattern that was distinct from both untreated

control MDCK cells (Figure 7) and the serum deprived MDCK(- Bcl-2) cell sample (Figure 5a), therefore not meeting the criteria stated for the definition of apoptotic cells. Staining appeared to be localized to one or more masses within the nuclei of both MDCK cell types (Figure 8, arrowhead). It has been shown that cisplatin does not affect peroxidase activity (79) therefore the pattern observed could not be due to changes in peroxidase activity by cisplatin.

Diffuse background cytoplasmic staining was also noted in cisplatin treated samples. This may be due to leakage of DNA fragments out of the nucleus. It is difficult to determine whether such leakage is the result of cisplatin treatment, an artifact resulting from preparation of the sample or if it represents a true biological phenomenon associated with cell death. Background staining may also be due to insufficient blocking of endogenous peroxidase activity by hydrogen peroxide. Some peroxidase activity has been previously found to be associated with the endoplasmic reticulum of kidney tubules (80).

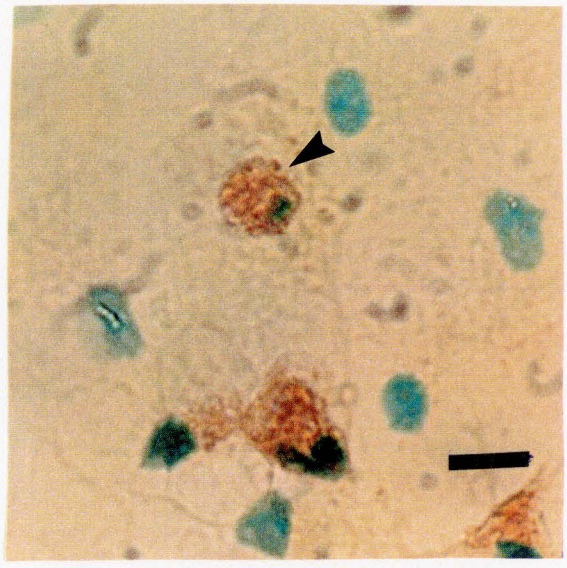
The results from the ISEL technique with cisplatin treatment of MDCK cells demonstrate that cisplatin did not induce apoptosis in these cells but did produce staining patterns that were similar in both control and Bcl-2 transfected MDCK cells.

Reduced methyl green staining of nuclei was also observed following cisplatin treatment. Methyl green is a nuclear stain which has been shown to bind selectively to AT rich regions of double stranded DNA (81-84). Optimal methyl green binding depends upon the high polymerized state of DNA and denaturation of DNA produces free, unbound methyl green and a subsequent decrease in staining (85). It has been suggested that the site-specific intrastrand crosslinks induced by cisplatin results in unwinding of the DNA (86). This may explain the reduction in methyl green staining of MDCK cells following cisplatin treatment.

Figure 5: ISEL technique showing features associated with apoptosis following serum deprivation for 72 hours

MDCK cells were cultured in 0.1% serum supplemented media for 72 hours. MDCK(- Bcl-2) cells are shown in a) and MDCK(+ Bcl-2) cells in b). The counterstain used was methyl green. Original magnification: 100X. Bars represent 20μM. Arrowhead indicates staining due to ISEL of fragmented DNA.

a)



b)

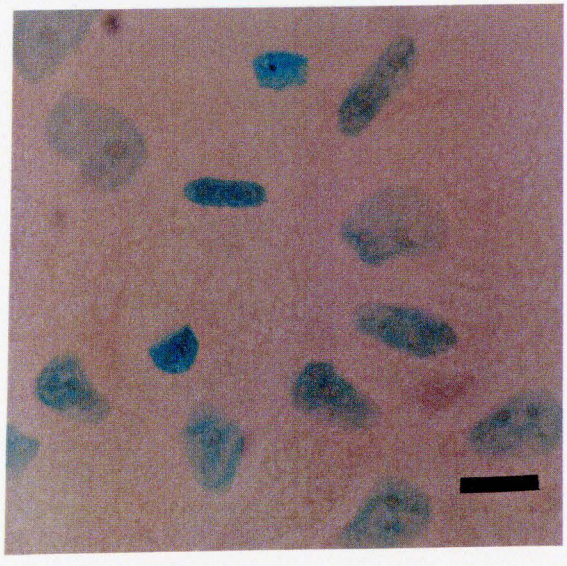
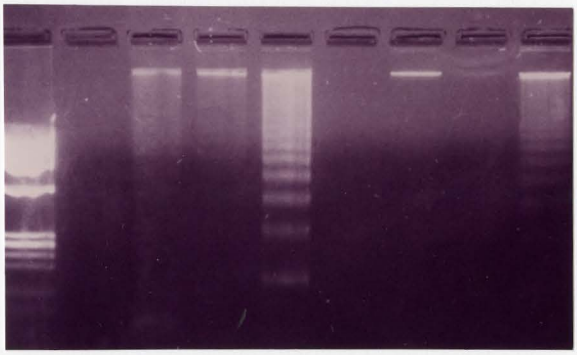


Figure 6: Serum deprived ISEL samples analyzed for DNA fragmentation

A nucleosomal DNA ladder is observed with MDCK(- Bcl-2) cells grown in 0.1% serum supplemented media for both 48 (lane 1, 2) and 72 hours (lane 3). In contrast, serum deprived MDCK(+ Bcl-2) cells do not display DNA ladder fragmentation after 48 hours (lanes 4-5). After culturing MDCK (+ Bcl-2) cells in 0.1% serum supplemented media for 72 hours, a faint DNA ladder appears (lane 6).


M
1
2
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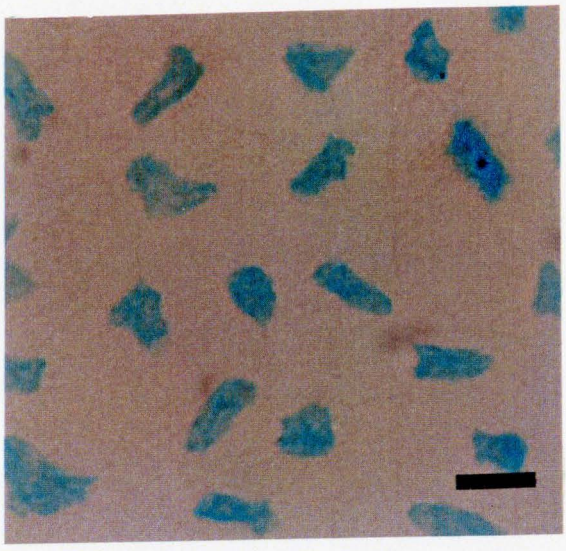
6

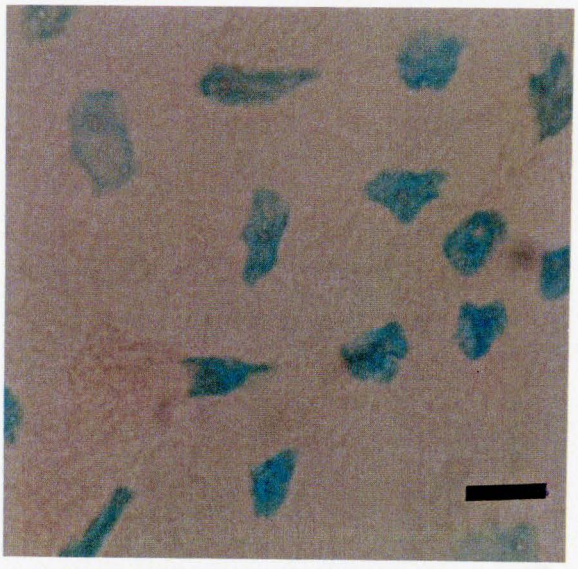
Figure 7: Controls used in the ISEL technique

ISEL was performed on MDCK cells with TdT absent from the labelling mix; MDCK(- Bcl-2) cells shown in a) and MDCK(+ Bcl-2) in b). MDCK cells untreated and grown in complete media for 48 hours were also subject to labelling; MDCK(- Bcl-2) cells shown in c) and MDCK(+ Bcl-2) in d). Bars represent 20μM. The counterstain used was methyl green. Original magnification: 100X.

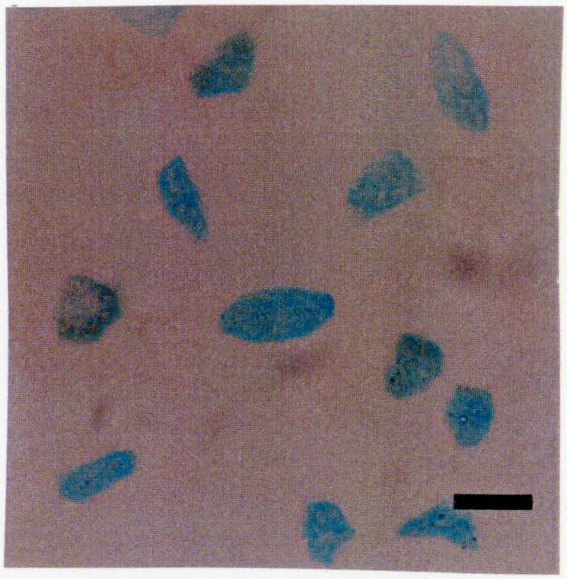
a)



b)



c)



d)

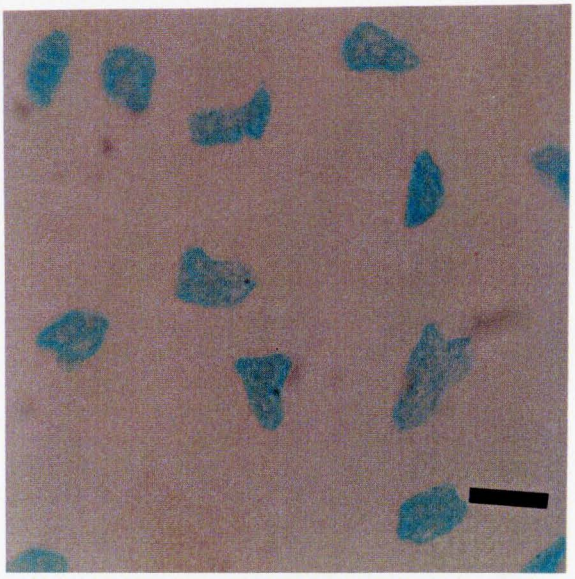
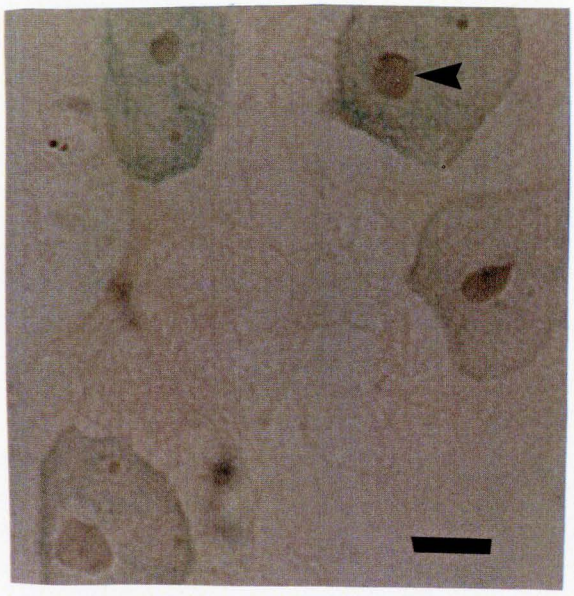


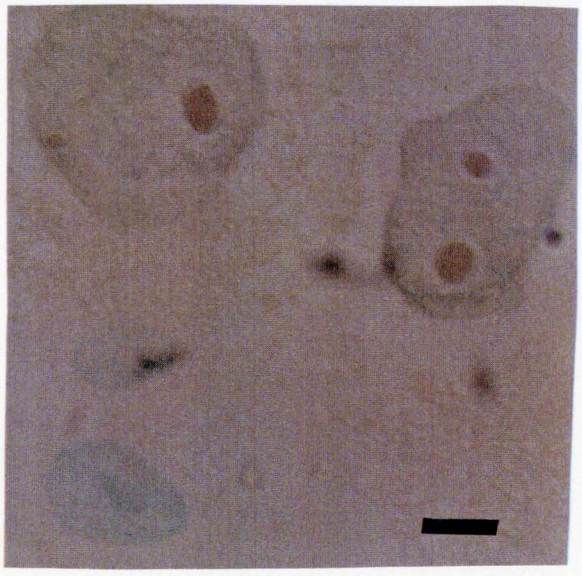
Figure 8: ISEL technique performed on MDCK cells following cisplatin treatment

MDCK cells were treated for 2 hours with 10μM cisplatin and then grown in fresh, drug free media for 72 hours. MDCK(- Bcl-2) cells are shown in a) and MDCK(+ Bcl-2) cells in b). The counterstain used was methyl green. Original magnification: 100X. Bars represent 20μM. Arrowhead indicates peroxidase staining.

a)



b)



ELECTRON MICROSCOPIC EXAMINATION OF MDCK CELLS

The morphological features associated with apoptosis were originally described by Wyllie et al (5). These morphological changes were observed in MDCK(- Bcl-2) cells following serum deprivation for 48 hours. Nuclear changes included chromatin condensation into a dense mass of nuclear material within a well preserved nucleus (Figure 9A, N). Concomitant with these nuclear changes, severe cytoplasmic condensation associated with the development of translucent cytoplasmic vacuoles was also observed (Figure 9A, V). Mitochondria (Figure 9A, arrow) and plasma membrane (Figure 9A, arrowhead) remain intact and loss of microvilli was also observed. In contrast, MDCK(+ Bcl-2) cells serum deprived for 48 hours (Figure 9B) appeared morphologically similar to untreated controls (Figure 9C) with relatively large nuclei, a prominent nucleoli and well preserved cytoplasmic organelles. These features are consistent with other descriptions of the normal morphology of MDCK cells (61). DNA analysis by gel electrophoresis confirmed the above results (Figure 10).

Treatment with cisplatin produced similar changes in both MDCK cell types. Extensive cytoplasmic vacuolization accompanied by condensation of the cytoplasm was consistently observed (Figure 11) and the dissolution of cytoplasmic organelles was clearly visible with longer post treatment incubation periods (Figure 11B, bottom). With early incubation periods of 48 hours, mitochondria still appeared intact (Figure 11A, m). The cytoplasmic changes observed are suggestive of cell death, likely by necrosis, but confirmation would require comparison with an appropriate necrotic control. Early peripheral nuclear condensation was observed (Figure 11A, bottom) however, in general the nuclear changes were not as evident as those of the serum deprived MDCK(- Bcl-2) sample. Cells retained an intact plasma membrane (Figure 11, arrowhead) in agreement with trypan blue exclusion data. Following a 2 hour exposure to cisplatin and a culturing period of 48 hours, 80% of the MDCK(- Bcl-2) and 78% of the MDCK 4.8(+ Bcl-2) cells still exclude trypan blue. Samples for electron microscopy included a combination of both adherent and nonadherent

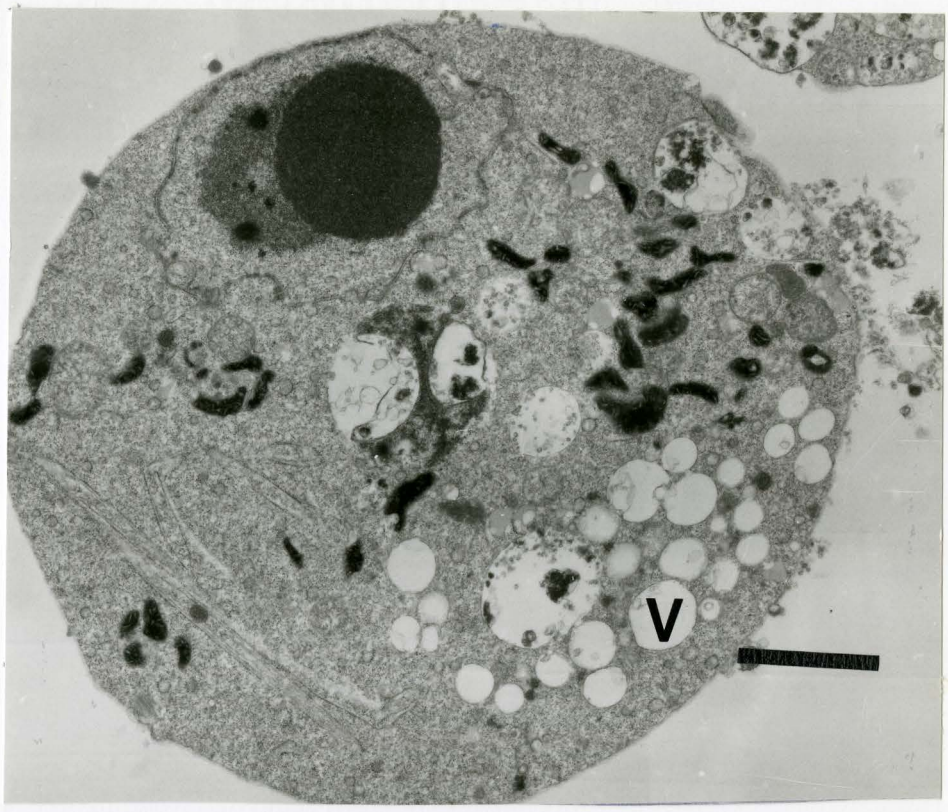
cells which may explain the viability data obtained or the changes observed may simply precede the loss of plasma membrane integrity.

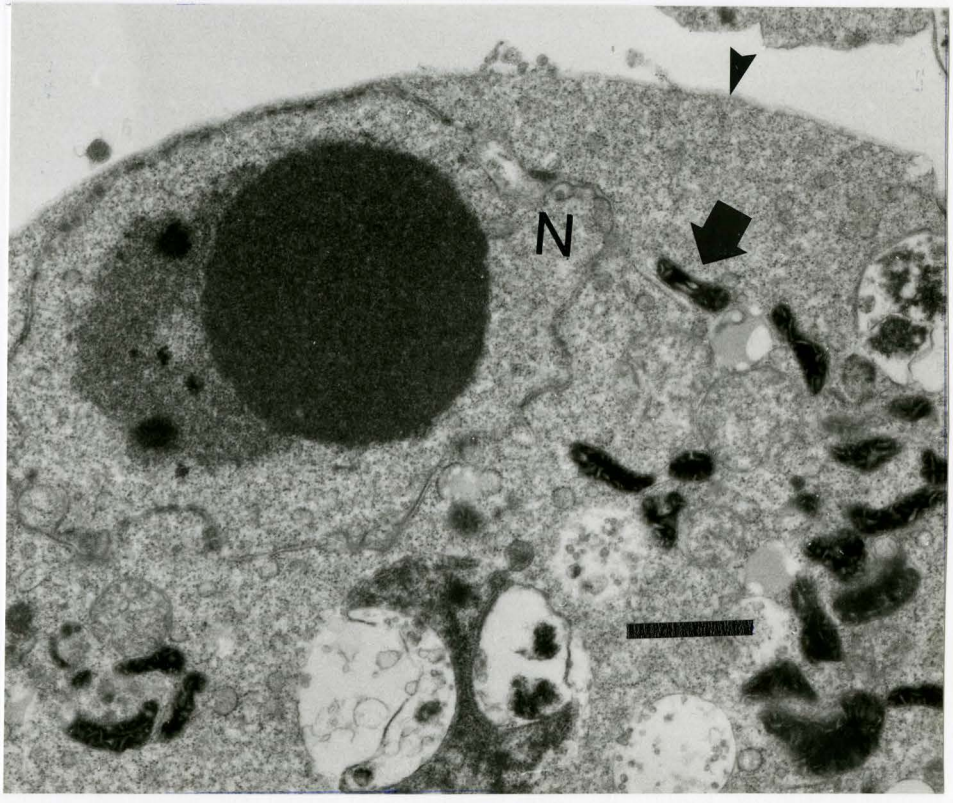
Figure 9: Ultrastructural features associated with apoptosis

Samples analyzed by electron microscopy included both adherent and nonadherent cells. To study the morphological changes associated with apoptosis, MDCK(- Bcl-2) cells (Figure 9A) were grown in 0.1% serum supplemented media for 48 hours at which time cells were collected, then fixed, stained and processed for electron microscopy as described in Materials and Methods. The electron micrograph shown in the bottom panel of Figure 9A, represents a magnification of the nucleus above. Scale bar: 1μM. (N) represents an apoptotic nuclei displaying chromatin condensation. (V) represents cytoplasmic vacuoles, the arrow indicates intact mitochondria and the arrowhead indicates an intact plasma membrane .

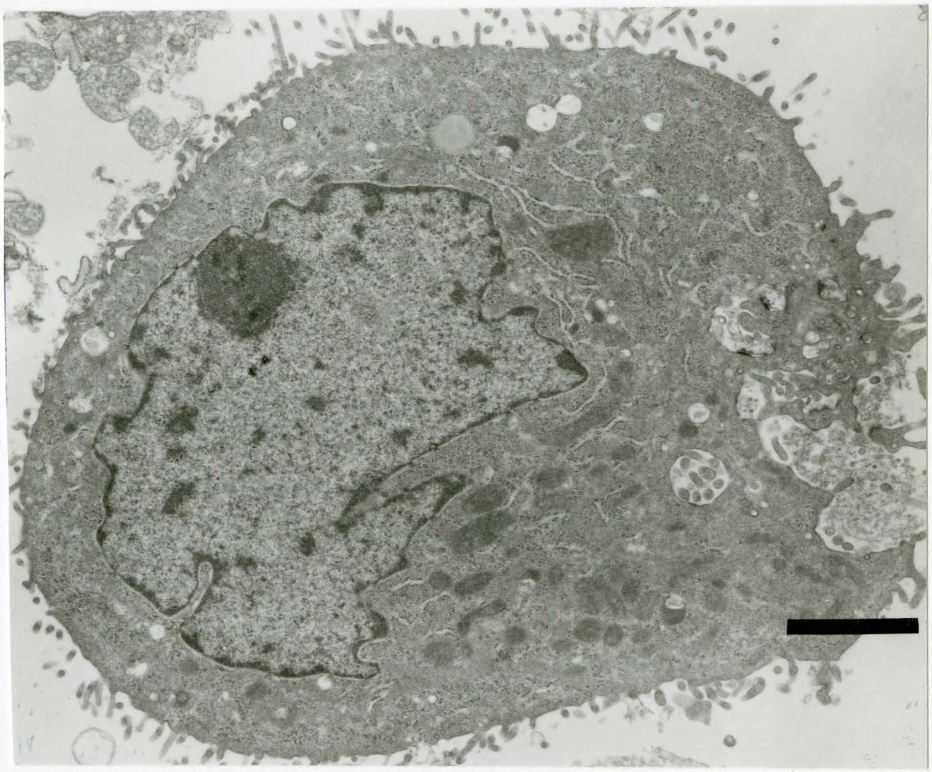
Morphological changes in MDCK(+ Bcl-2) cell sample grown under similar conditions were also studied (Figure 9B). MDCK cells grown in complete media for 48 hours were included as untreated control samples (Figure 9C; top - MDCK(- Bcl-2); bottom - MDCK (+ Bcl-2). Scale bar: 2μM.

A

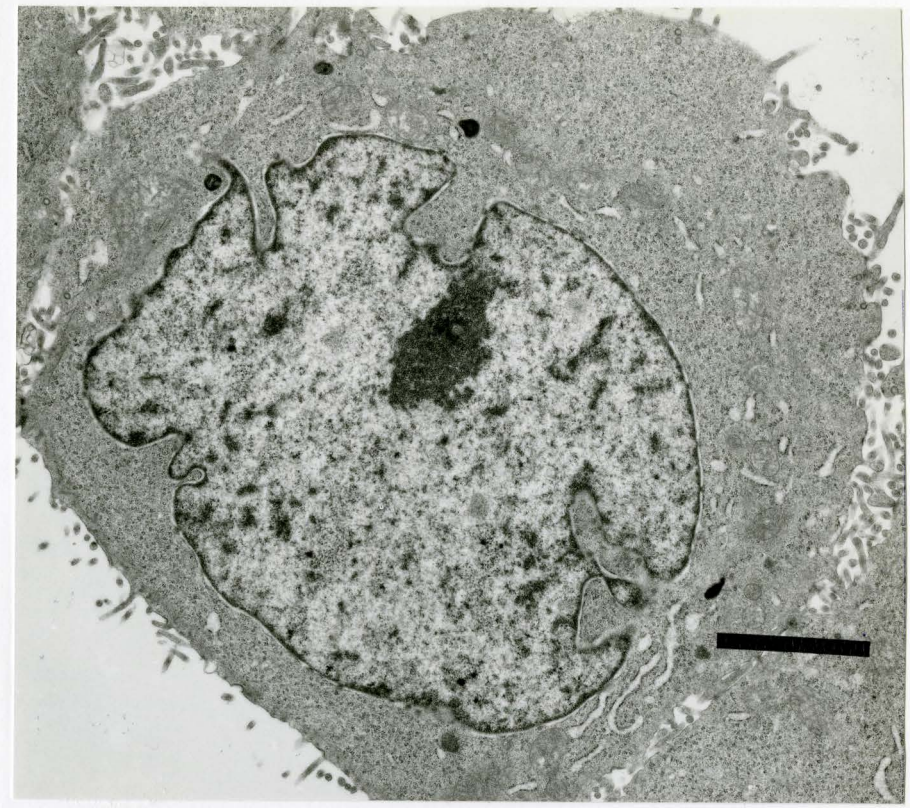




B



C



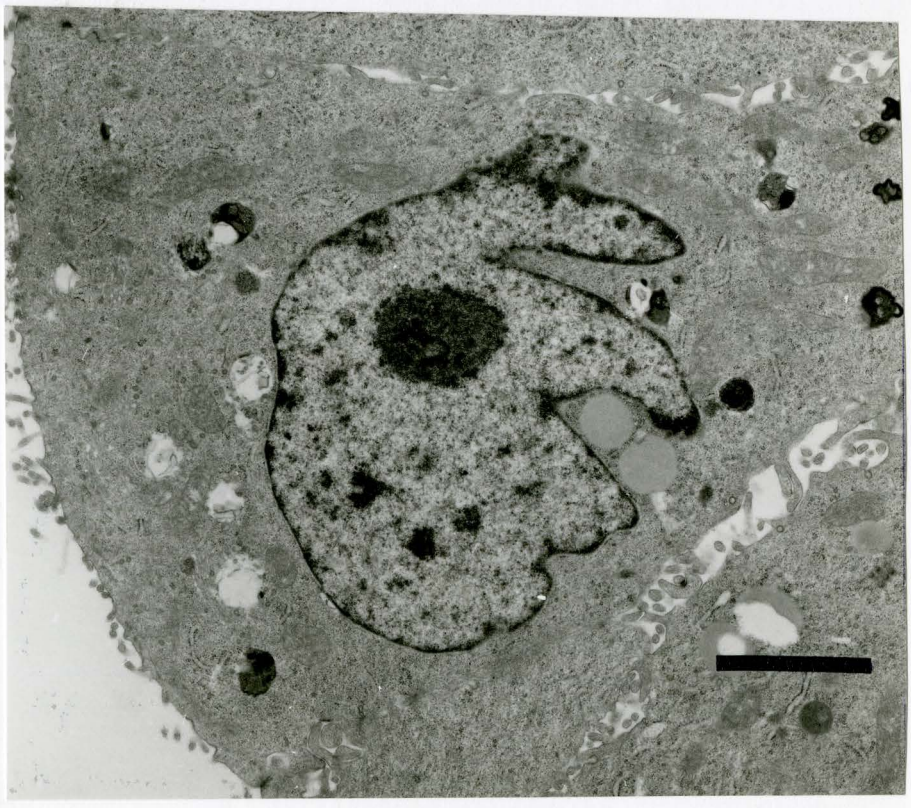


Figure 10: DNA analysis of samples prepared in duplicate with the electron microscopy study

DNA was extracted from MDCK(- Bcl-2) cells grown in 0.1% serum supplemented media for 48 hours (lane 1) and 72 hours (lane 2) and from MDCK(+ Bcl-2) cells grown under similar conditions for 48 hours (lane 3) and 72 hours (lane 4) and run on a 1.5% agarose gel containing 0.5μg/ml of ethidium bromide for 2 hours at 75V. DNA analyzed from untreated MDCK cells is shown in (C). The ϕX174 RF DNA/Hae III fragments is shown in (M).

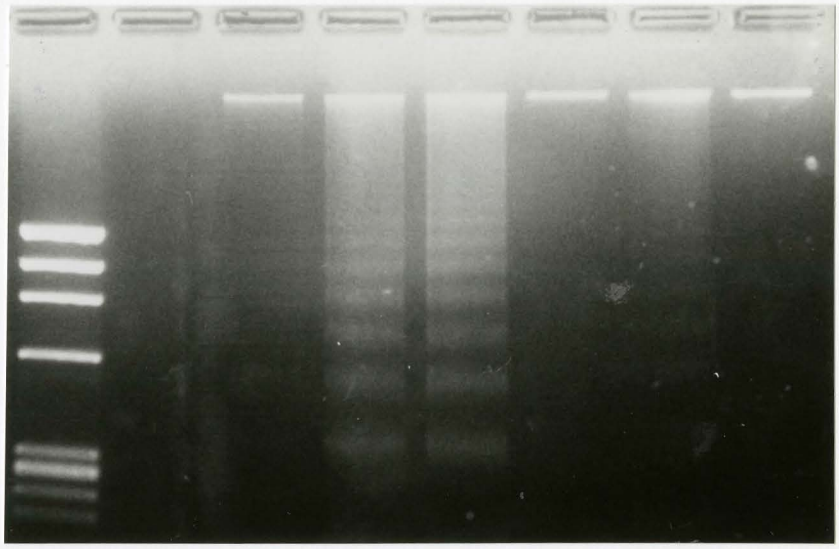
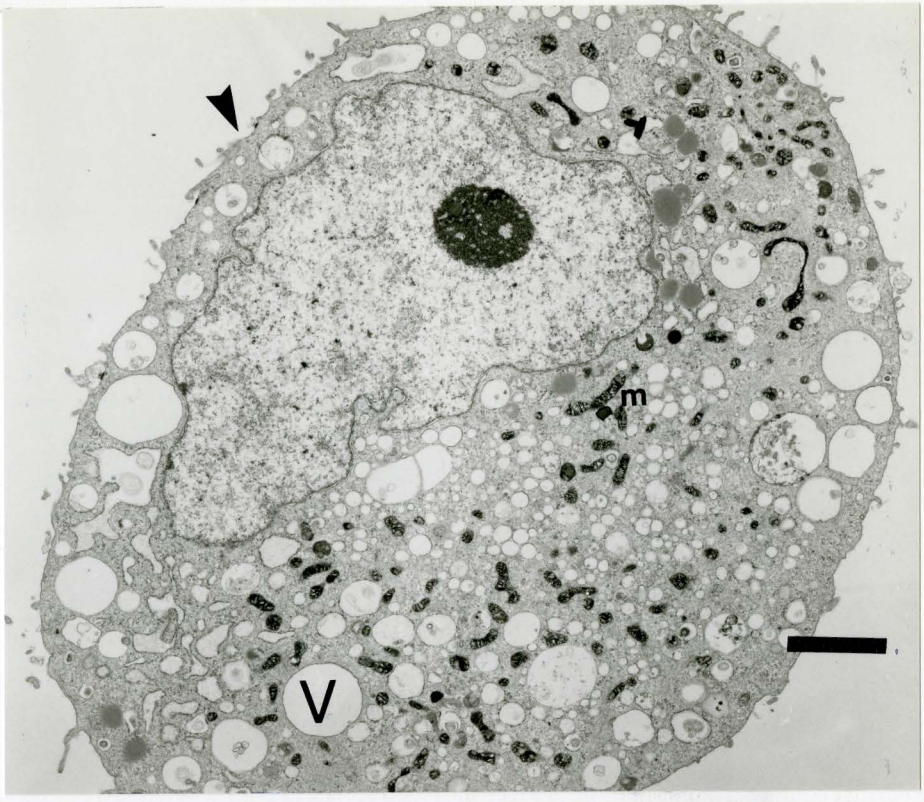
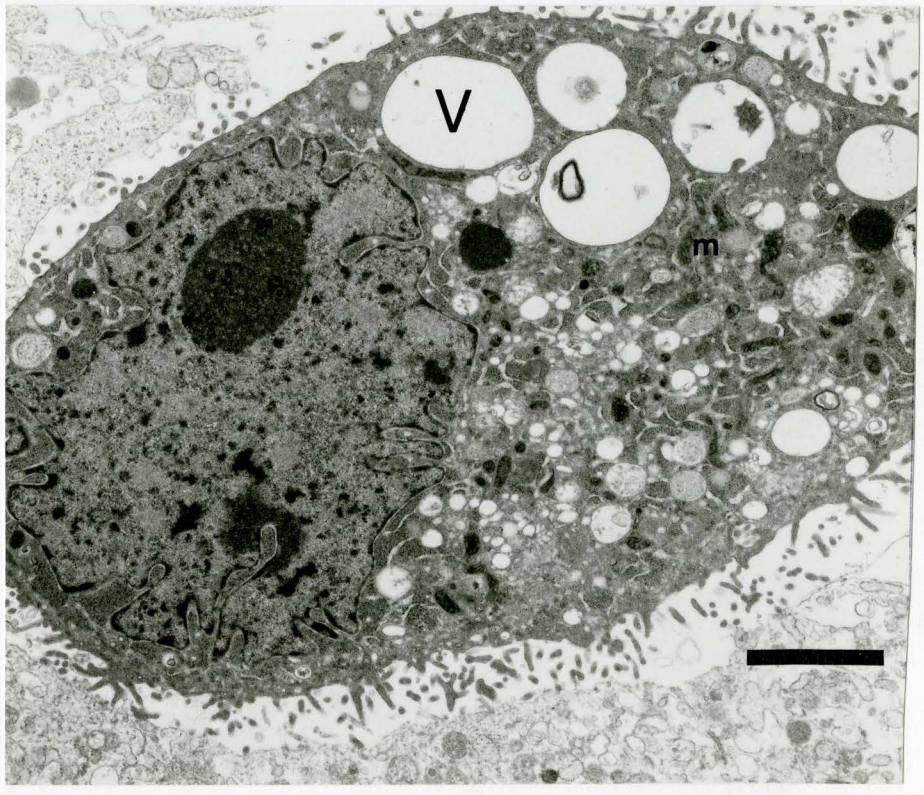

M
C
1
2
3
4
C

Figure 11: Morphological changes induced by cisplatin (10μM)

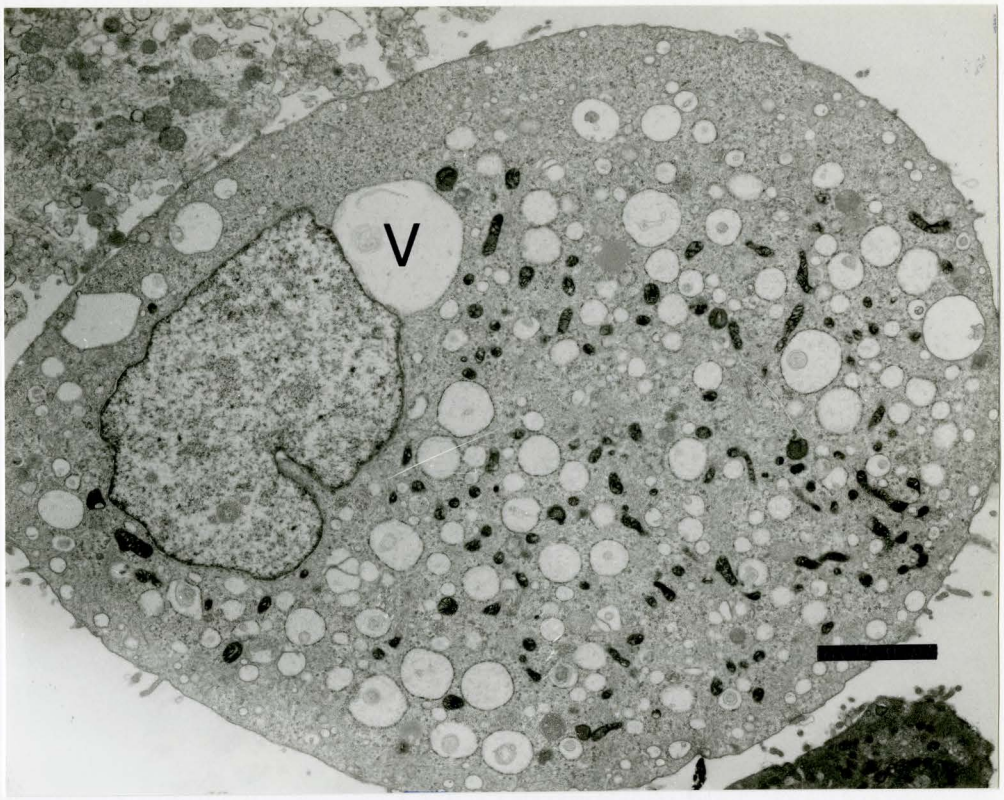
MDCK cells were incubated with cisplatin for 2 hours and then cultured in drug free media for 48 (Figure 11A) or 72 hours (Figure 11B). Adherent and nonadherent cells were collected, then fixed, stained and processed by electron microscopy as described in Materials and Methods. Ultrastructural features of MDCK(- Bcl-2) cells are shown in the top panels and MDCK(+ Bcl-2) cells exposed to identical conditions are shown the bottom panels. Scale bar: 2μM. (V) represents cytoplasmic vacuoles and (m) indicates mitochondria. Arrowhead indicates intact plasma membrane.

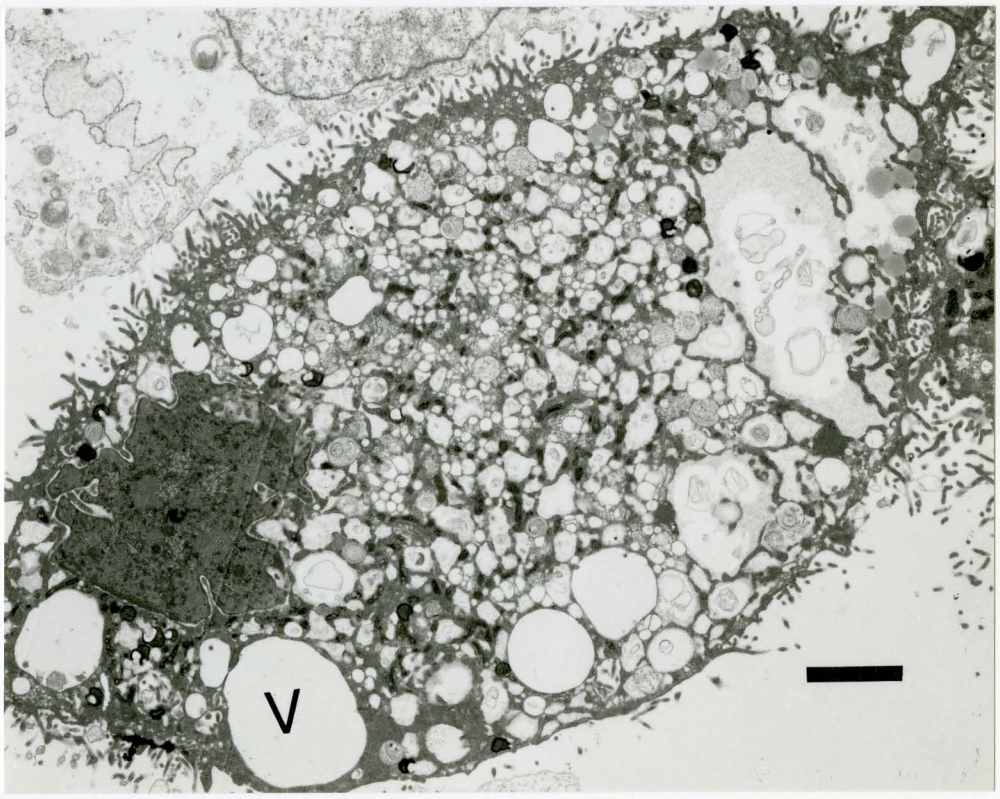
A





B





BCL-2 EXPRESSION DOES NOT ENHANCE SURVIVAL IN THE PRESENCE OF CISPLATIN

The survival response of the MDCK cells to varying cisplatin doses was evaluated using two different survival assays. In the DNA fluorometric assay, measurement of cell survival is based upon the determination of DNA content. Cells included in this measurement are only those which remain adhered to the wells; cells that have detached from the wells are excluded. Data from this assay show that with increasing cisplatin doses no difference in cell survival between MDCK(- Bcl-2) and MDCK(+ Bcl-2) cells is observed over the intervals of 2,3,4 and 5 days (Figure 12) . Over the 3-5 day period, similarity in response of both cell types to cisplatin cytotoxicity is particularly evident (Figure 12b-d).

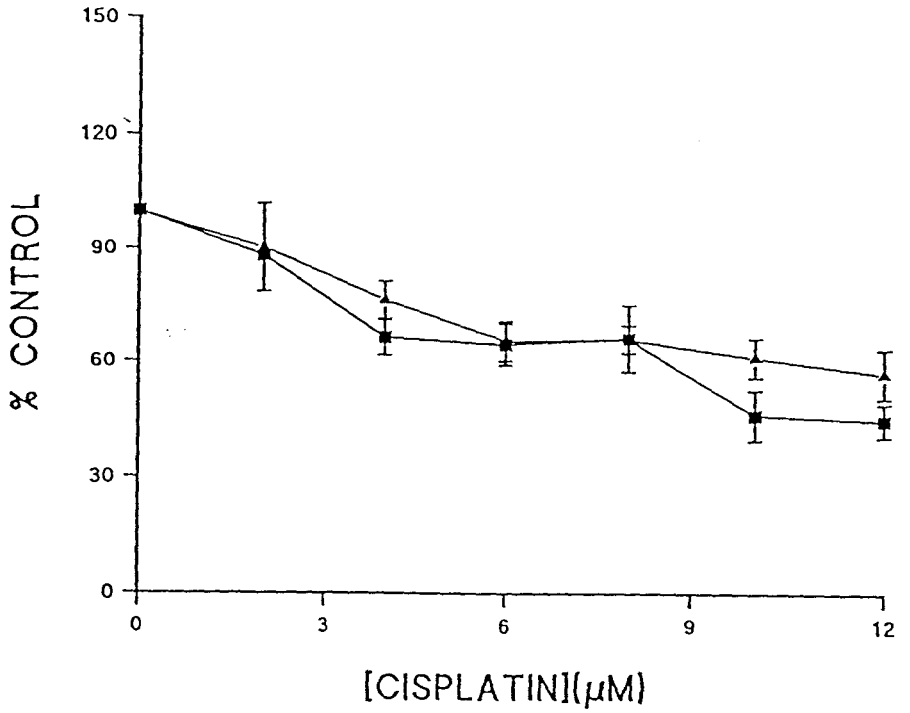
The second assay used to assess survival was the colony forming assay. This assay provides information regarding the ability of a cell to proliferate and form colonies following exposure to drug. A time period of at least 6 days is required in order to maintain a reasonable count in both the control (no drug treatment) cell population and in the population treated with the highest drug dose. Data from

the clonogenic assay also demonstrates that no difference in proliferative capacity between MDCK(- Bcl-2) and MDCK(+ Bcl-2) cells exists (Figure 13). A comparison of LD_{50} and LD_{75} values for both MDCK(- Bcl-2) and MDCK(+ Bcl-2) cells further illustrates the similarity in response to cisplatin in both cell types (Table 3). It is evident that the expression of Bcl-2 does not protect MDCK cells from the cytotoxic effects of cisplatin.

FIGURE 12: Effects of cisplatin on MDCK cell survival over varying periods of time

Cytotoxicity was assessed by the DNA fluorometric assay following a 2 hour cisplatin exposure after which time cells were grown in fresh, drug free media for desired time periods. The time periods chosen following removal of drug include: a) 2 days, b) 3 days, c) 4 days and d) 5 days. Values are expressed as a percentage of control ± SD and represent data from 3 independent experiments done in duplicate. (■)MDCK(- Bcl-2); (▲)MDCK(+ Bcl-2)

a)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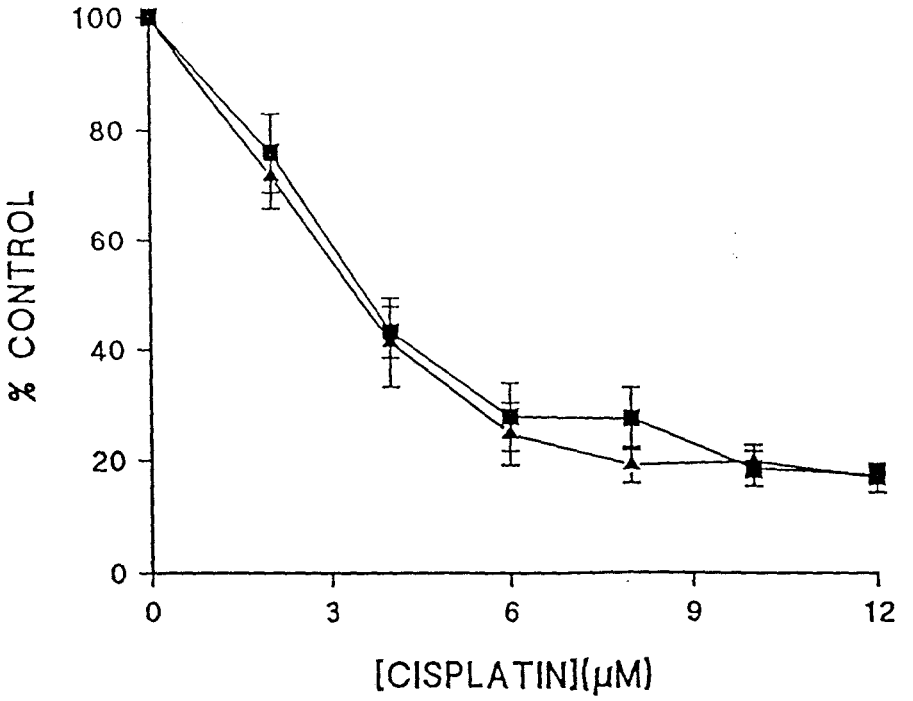
[CISPLATIN](μM)

b)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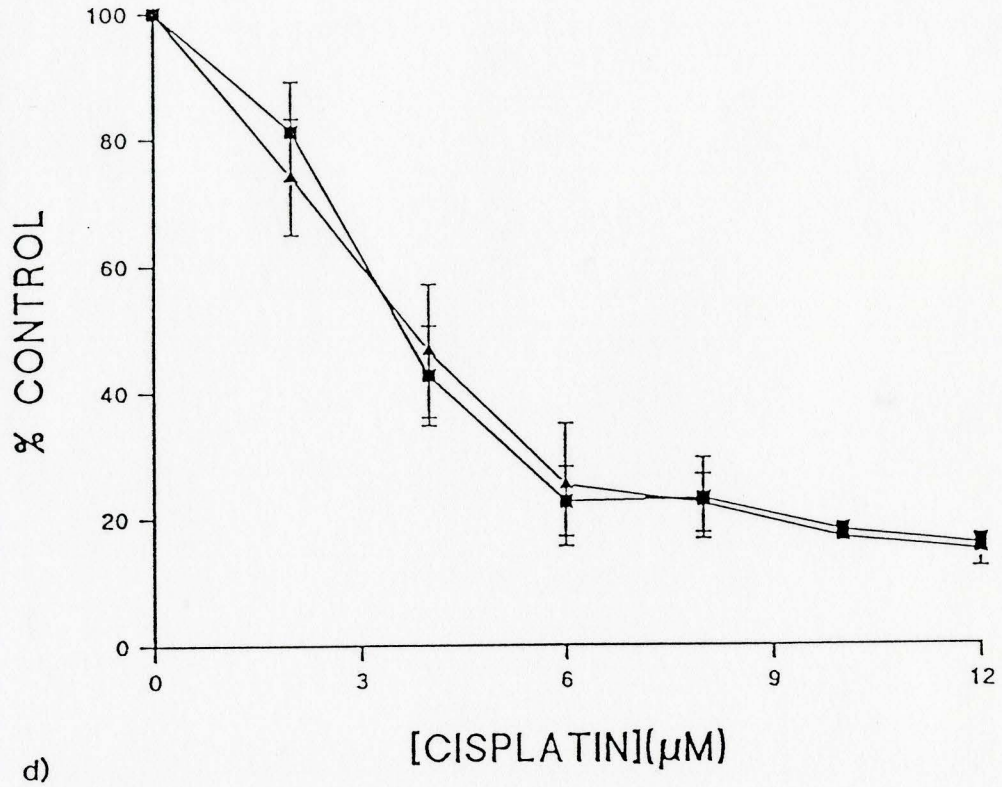
c)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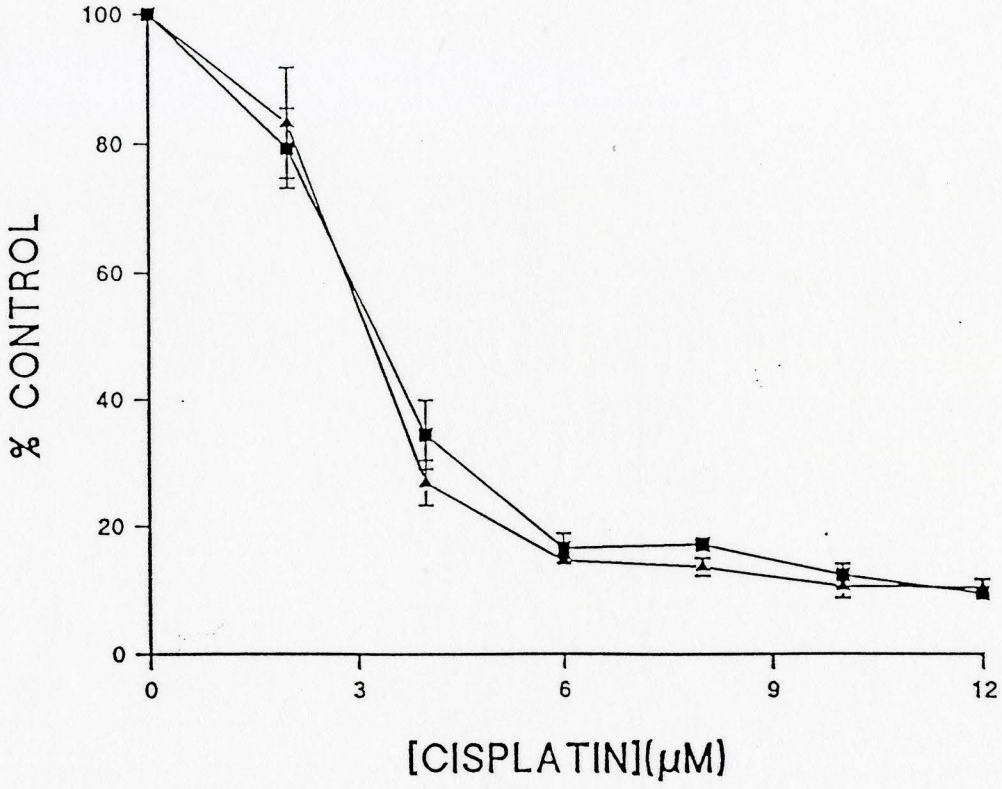
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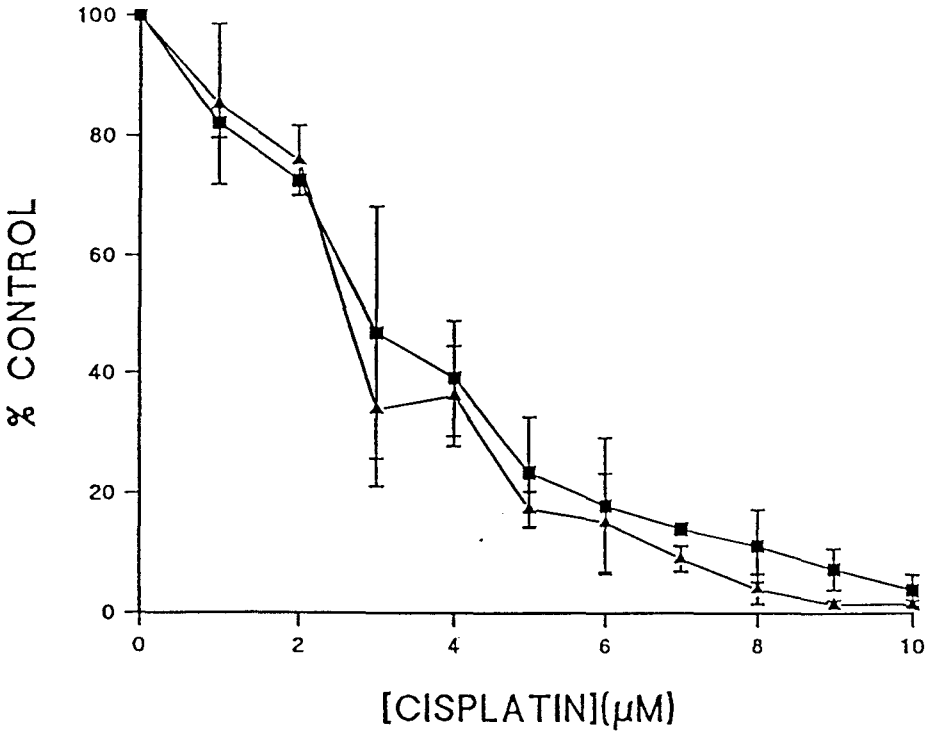
[CISPLATIN](μM)

FIGURE 13: Effects of cisplatin on the proliferation of MDCK cells

Cytotoxicity was assessed by clonogenic survival after a 2 hour cisplatin exposure after which time cells were grown in fresh, drug free media for desired time periods. The time periods chosen following removal of drug include: a) 6 days and b) 7 days. Values are expressed as a percentage of control ± SD and represent data from 2 independent experiments done in triplicate. (■)MDCK(- Bcl-2); (▲)MDCK(+ Bcl-2) .

a)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8
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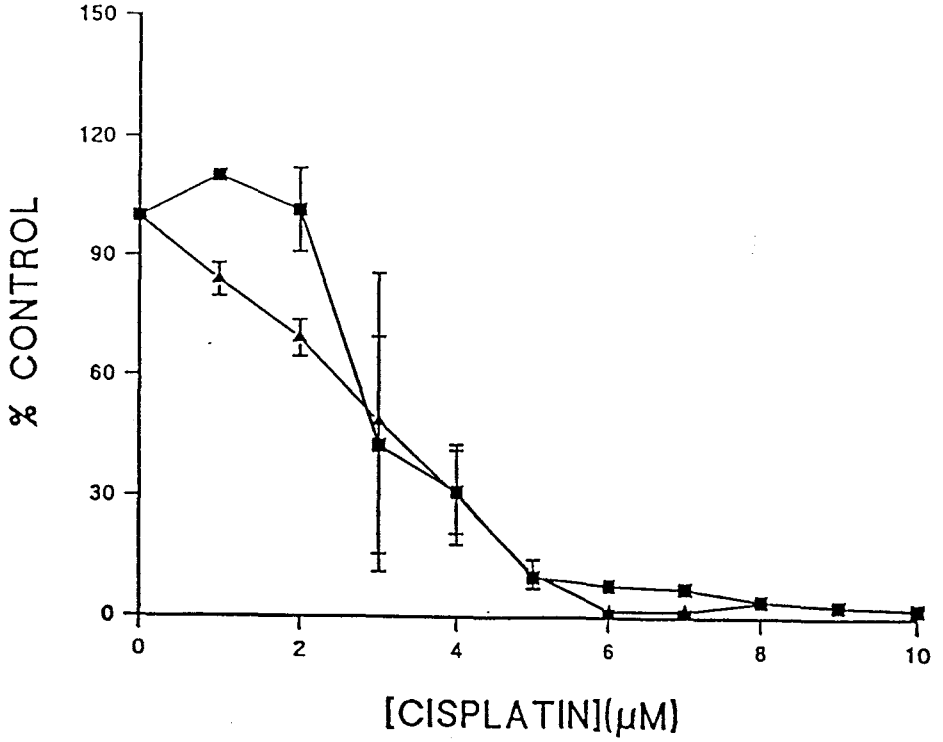
[CISPLATIN](μM)

b)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[CISPLATIN](μM)

TABLE 3: A COMPARISON OF *LD_{50} AND *LD_{75} VALUES FOR BOTH MDCK(- Bcl-2) AND MDCK(+ Bcl-2) CELLS

POST INCUBATION TIME FOLLOWING A 2HR DDP PULSE		4dys	5dys	6dys	7dys
MDCK(- Bcl-2)	LD_{50}	3.9 ± 0.8	3.4 ± 0.4	2.7 ± 0.4	3.6 ± 0.02
	LD_{75}	7.2 ± 0.8	5.9 ± 0.3	4.4 ± 0.7	4.5 ± 0.02
MDCK(+ Bcl-2)	LD_{50}	3.7 ± 1.0	3.3 ± 0.4	2.7 ± 0.1	2.2 ± 0.4
	LD_{75}	7.1 ± 1.3	5.7 ± 0.1	3.9 ± 0.2	3.2 ± 0.4

Cisplatin doses at which 50% (LD_{50}) and 25% (LD_{75}) of the cell population survives was obtained using the Median Effect Analysis which determines the dose for any given effect from a median effect plot (87). Values represent data from 3 independent experiments done in duplicate and is expressed as the mean ± SD.

TABLE 4: DATA FROM A REPRESENTATIVE 7 DAY COLONY FORMING ASSAY

MDCK(- Bcl-2)											
[CISPLATIN]μM	0	1	2	3	4	5	6	7	8	9	10
# OF COLONIES	31	40	31	18	12	5	4	1	1	2	1
	38	36	29	32	19	2	3	5	1	1	0
	30	33	30	19	10	4	0	0	1	0	0
AVG	33	36	30	23	13.7	3.7	2.3	2.3	1.00	1.00	0.33
± STD	3.6	2.9	0.8	6.4	3.9	1.2	1.7	1.9	0.00	0.8	0.5
MDCK(+ Bcl-2)											
# OF COLONIES	32	29	20	30	11	5	1	0	1	0	0
	32	25	21	31	17	5	0	1	1	0	0
	20	20	21	20	8	2	0	0	1	0	0
AVG	28	25	21	27	12	4	0.3	0.3	1	0	0
± STD	5.7	3.7	0.5	4.97	3.7	1.4	0.5	0.5	0	0	0

CHAPTER FOUR

DISCUSSION

Drug induced apoptosis has been demonstrated in a number of different cell types and appears to be a dose dependent phenomenon (7,88,89). Cisplatin represents a model chemotherapeutic agent capable of inducing apoptosis in a manner consistent with that of the classical thymocyte model in a variety of cell types. Subsequent studies demonstrated that Bcl-2 overexpression protects cells from cisplatin induced apoptosis, implicating a role for Bcl-2 in mediating chemotherapeutic drug resistance (90,91). Recent evidence suggests that the ability to induce apoptosis is cell type-specific (30,92,93), challenging the generalization of drug induced apoptosis across all cell types. The varied observation among different cell types to undergo apoptosis by cytotoxic agents may be explained by differences in cell phenotype and the nature of the cytotoxic agent used. The evidence presented in this thesis demonstrates that in one cell model, which

possesses a functional apoptotic pathway, activation of this pathway and subsequent protection by Bcl-2 is dependent on the nature of the cytotoxic agent used. This suggests that multiple pathways to cell death exist which are differentially regulated by Bcl-2.

Normal mammalian cells require growth factors for survival as demonstrated by the need for serum in cell culture and when deprived of such factors cells are driven into a non-proliferative state and eventually undergo cell death. Induction of apoptosis following serum deprivation has been demonstrated in several cell types (94-98). The specific factors required for MDCK growth have been isolated by demonstrating growth of MDCK cells in hormone supplemented, serum free media (99). The evidence presented in this thesis demonstrates that MDCK(- Bcl-2) cells cultured in low serum conditions die by apoptosis.

MDCK(- Bcl-2) cells cultured in 0.1% serum supplemented media for 48 hours lost cell to cell contact, detached from tissue culture plates on which they were grown and became rounded. These features are consistent with those observed with other serum deprived

cells. DNA extracted from serum deprived MDCK(- Bcl-2) cells displayed the classical "DNA ladder" when separated by gel electrophoresis (Figure 3A-D,F,I-J), demonstrating that serum deprivation induced apoptosis in this cell type.

Further support for the induction of apoptosis was obtained from employing a technique based on the in situ end labelling of DNA strand breaks (ISEL). ISEL experiments were used to identify apoptotic cells on a cell to cell basis, combining both the biochemical characterization of DNA fragmentation with typical morphology. MDCK(- Bcl-2) apoptotic cells exhibited nuclear staining consistent with peripheral chromatin condensation and DNA fragmentation (Figure 5a). Similar in situ results have been observed in other models of serum deprivation induced apoptosis (100).

The morphological changes associated with apoptosis in these cells were examined by electron microscopy. MDCK(- Bcl-2) cells cultured in reduced serum conditions displayed chromatin condensation producing a dense mass of nuclear material within a well preserved nucleus. Cytoplasmic condensation produced numerous

vacuoles, however mitochondria and the plasma membrane of MDCK(- Bcl-2) cells remained intact (Figure 9A). These results agree with other electron microscopic analysis of apoptotic MDCK cells (62). In all of the above assays MDCK(+ Bcl-2) cells cultured in 0.1% serum supplemented media produced results identical to the untreated control population, demonstrating that the overexpression of Bcl-2 protects against serum deprivation-induced apoptosis (Figure 9B). The induction of apoptosis following serum deprivation in MDCK(- Bcl-2) cells is not surprising since floating apoptotic cells have been found in confluent cultures where competition for the limiting amounts of available survival factors and a reduced accessibility for matrix attachment may trigger apoptosis (101).

The assays described above were also performed on MDCK cells treated with varying concentrations of cisplatin. In the nucleosomal DNA ladder assay, both MDCK cell types were treated with cisplatin for two hours to mimic clinical treatment protocols. Studies with cisplatin in other cell models have shown that DNA ladder formation is both time and dose dependent (28,89) therefore, a range

of post treatment periods (incubation in drug free media) from 8 to 48 hours were investigated. DNA analysis of MDCK cells treated with varying cisplatin doses did not result in DNA ladder formation (Figure 3). Increasing toxicity by culturing MDCK cells for 72 hours post treatment still did not induce DNA ladder formation (Figure 4). It appears that cisplatin does not induce apoptosis in MDCK cells and therefore subsequent protection by Bcl-2 is not expected.

Examples of cisplatin induced apoptosis in the absence of DNA ladder formation have also been described, where DNA from these cells was degraded into larger fragments (14). High molecular weight DNA degradation associated with apoptosis has been well characterized (102-104). It has been suggested that perhaps not all cell types possess the endonuclease involved in "classical" apoptosis (apoptosis with DNA ladder formation) or that this endonuclease may be constitutively present in some cells while inducible in others (105). To identify other features associated with apoptosis, additional apoptotic assays were applied to cisplatin treated samples.

ISEL experiments produced a unique staining pattern that was identical in both MDCK cell types but distinct from the pattern observed following serum deprivation (Figure 7). These results further support the notion that cisplatin does not induce apoptosis and suggests that the effects of cisplatin were not abrogated by the overexpression of Bcl-2. In principle, the ISEL technique detects all DNA strand breaks and such nonspecific staining of nuclei has been previously identified as a limitation of ISEL techniques (106-108). While cisplatin by itself does not induce strand breaks, it may induce processes to remove damaged DNA, producing repair intermediates that can be effectively labelled by ISEL (109,110). In addition, ISEL may also label the randomly occurring DNA fragmentation associated with necrosis (111-113). To classify the staining pattern observed as any one of the above would require the use of specific controls for staining due to DNA damage repair intermediates and necrosis.

The ultrastructural change consistently observed in both MDCK cell types following cisplatin treatment was extensive cytoplasmic vacuolization accompanied by cytoplasmic condensation (Figure 11).

Studies which assess the nephrotoxic effects of cisplatin have also noted vacuolization specifically in the cells of the distal tubule (114). The vacuoles observed may be due to the activation of lysosomes. Cisplatin treatment has been shown to preferentially increase the number of lysosomes in the kidney and electron microscopy revealed the release of hydrolytic enzymes from lysosomes into the cytoplasm leading to complete lysis of the cell and eventually cell death (115). Whether the vacuoles observed in MDCK cells imply a role for lysosomes requires further investigation using appropriate histochemical assays. Changes in nuclei were difficult to assess since they appeared relatively similar to the otherwise normal, untreated MDCK nuclei (compare Figure 9C).

In general, the ultrastructural changes observed are suggestive of cell death, likely by necrosis. This is particularly evident in MDCK cells cultured for prolonged periods following treatment (Figure 11B). It has been proposed that agents which induce apoptosis may also induce necrosis depending on the cell type and the extent of cellular injury. Clinically, nephrotoxicity has been recognized as the dose

limiting factor associated with the use of cisplatin and acute tubular necrosis is often a prominent feature of cisplatin nephrotoxicity (116-118). Therefore, the notion that cisplatin treatment induces necrosis in MDCK cells is a reasonable explanation. Necrosis is often detected as a smear of random DNA fragments following gel electrophoresis. This was not observed with MDCK cells following treatment with cisplatin (Figure 3). However, necrosis has also been associated with high molecular weight DNA fragmentation (119) and this may explain the results obtained in Figure 3. Interestingly, overexpression of Bcl-2 did not protect against cisplatin toxicity, suggesting that Bcl-2 does not protect against all forms of cell death.

It has been repeatedly shown that the overexpression of Bcl-2 correlates with resistance to a wide variety of chemotherapeutic drugs, based on survival measurements evaluated by trypan blue exclusion (91). In this thesis, a thorough examination of MDCK cell survival in the presence of increasing cisplatin was investigated using two different survival assays. Elevated Bcl-2 levels did not alter survival in response to cisplatin treatment.

Bcl-2 overexpression did not affect survival over shorter time intervals (Figure 12) suggesting that in this model Bcl-2 expression was insufficient in preventing or delaying drug induced death. A delay in drug induced cell death by Bcl-2 has been associated with increasing the time for repair to take place and therefore allowing a proportion of cells within a population to survive and to continue to proliferate following drug treatment (120). Such an effect would depend on several variables, including the inherent rate of repair in a particular cell type. It has already been shown that kidney cells are exquisitely sensitive to cisplatin, this likely accounts for the rapid decline observed in MDCK survival following drug treatment and the absence of Bcl-2 protection.

Clonogenic assays were used to study the long term growth response of MDCK cells to cisplatin. It should be noted that to account for the differing growth properties of MDCK(- Bcl-2) and MDCK(+ Bcl-2) cells, survival was expressed as a percentage of control (untreated samples for both cell types). Increased levels of

Bcl-2 did not confer a long term growth advantage to MDCK cells (Figure 13).

In conclusion, data presented in this thesis supports the hypothesis that multiple pathways to cell death exist within a particular cell type (96,121), activation of apoptosis is dependent upon the stimuli used and such pathways are differentially regulated. The data presented strongly suggests that cisplatin treatment overides the apoptotic pathway present in MDCK cells and as a result subsequent protection against cisplatin cytotoxicity by Bcl-2 was not observed. Serum deprivation, however, functions as an appropriate apoptotic "trigger" and as a result the apoptotic pathway activated was sufficiently regulated by Bcl-2 expression.

REFERENCES

1. Inversen OH. Kinetics of Cellular Proliferation and Cell loss in Human Carcinomas. A Discussion of Methods Available for in vivo studies. European Journal of Cancer 1967; 3:389

2. Cliffton KH, Yatvin MB. Cell Population Growth and Cell Loss in the MTG-B Mouse Mammary Carcinoma. Cancer Research 1970; 30:658

3. Kerr JFR, Wyllie AH, Currie AR. Apoptosis: A Basic Biological Phenomenon With Wide-Ranging Implications in Tissue Kinetics. British Journal of Cancer 1972; 26:239-257.

4. Cotter TG, Lennon SV, Glynn JG, Martin SJ. Cell Death via Apoptosis and its Relationship to Growth, Development and Differentiation of both Tumor and Normal Cells. Anticancer Research 1990; 10:1153-1160.

5. Wyllie AH, Kerr JFR, Currie AR. Cell Death: The Significance of Apoptosis. International Review of Cytology 1980; 68:251-301.

6. Eastman A. Activation of Programmed Cell Death by Anticancer Agents: Cisplatin as a Model System. Cancer Cells 1990; 2:275-280.

7. Dive CA, Evans CA, Whetton AD. Induction of apoptosis-new targets for cancer chemotherapy. Seminars in Cancer Biology 1992; 3:417-427.

8. Hickman JA. Apoptosis induced by anticancer agents. Cancer and Metastasis Reviews 1992; 11:121-139.

9. Wyllie AH. Glucocorticoid-induced thymocyte apoptosis is associated with endogenous endonuclease activation. Nature 1980; 284:555-556.

10. Kaufmann H. Induction of endonucleolytic DNA cleavage in human acute myelogenous leukemia cells by etoposide, camptothecin and other cytotoxic anticancer drugs: a cautionary note. Cancer Research 1989; 49:5870-5878.

11. Walker PR, Smith C, Youdale T, Leblanc J, Whitfield JF, Sikorska M. Topoisomerase II-reactive chemotherapeutic drugs induce apoptosis in thymocytes. Cancer Research 1991; 51:1078-1085.

12. Gorczyca W, Gong J, Ardelt B, Traganos F, Darzynkiewicz Z. Cell Cycle Related Differences in Susceptibility of HL-60 Cells to Apoptosis Induced by Various Antitumor Agents. Cancer Research 1993; 53:3186-3192.

13. Miyashita T, Reed JC. bcl-2 Gene Transfer Increases Relative Resistance of S49.1 and WEH17.2 Lymphoid Cells to Cell Death and DNA Fragmentation Induced by Glucocorticoids and Multiple Chemotherapeutic Drugs. Cancer Research 1992; 52:5407-5411.

14. Ormerod MG, O'Neill CF, Robertson D, Harrap KR. Cisplatin Induces Apoptosis in a Human Ovarian Carcinoma Cell Line without Concomitant Internucleosomal Degradation of DNA. Experimental Cell Research 1994; 211:231-237.

15. Barry MA, Behnke CA, Eastman A. Activation of Programmed Cell Death (Apoptosis) by Cisplatin, other Anticancer Drugs, Toxins and Hyperthermia. Biochemical Pharmacology 1990; 40:2353-2362.

16. Evans DL, Dive CA. Effects of Cisplatin on the Induction of Apoptosis in Proliferating Hepatoma Cells and Nonproliferating Immature Thymocytes. Cancer Research 1993; 53:2133-2139.

17. O'Connor PM, Wassermann K, Sarang M, Magrath I, Bohr VA, Kohn KW. Relationship between DNA Cross-Links, Cell Cycle, and Apoptosis in Burkitt's Lymphoma Cell Lines Differing in Sensitivity to Nitrogen Mustard. Cancer Research 1991; 51:6550-6557.

18. Walton MI, Whysong D, O'Connor PM, Hockenbery D, Korsmeyer SJ, Kohn KW. Constitutive Expression of Human Bcl-2 Modulates Nitrogen Mustard and Camptothecin Induced Apoptosis. Cancer Research 1993; 53:1853-1861.

19. Yoshioka A, Tanaka S, Hiraoka O, Koyama Y, Hiroto Y, Ayusawa D. 5-Fluorodeoxyuridine-induced DNA double strand breaks in mouse FM3A cells and the mechanism of cell death. Journal of Biological Chemistry 1987; 262:8235-8241.

20. Gunji H, Kharbanda S, Kufe D. Induction of internucleosomal DNA fragmentation in human myeloid leukemia cells by 1-β-D-arabinofuranosylcytosine. Cancer Research 1991; 51:162-166.

21. Aldridge DR, Arends M, Radford I. Increasing the susceptibility of the rat 208F fibroblast cell line to radiation-induced apoptosis does not alter its clonogenic survival dose-response. British Journal of Cancer 1995; 71:571-577.

22. Warters RL. Radiation-induced Apoptosis in a Murine T-Cell Hybridoma. Cancer Research 1992; 52:883-890.

23. He X, Sikes RA, Thomsen S, Chung LW, Jacques SL. Photodynamic Therapy with Photofrin II Induces Programmed Cell

Death in Carcinoma Cell Lines. Photochemistry and Photobiology 1994; 59:468-473.

24. Loehrer J, Patrick., Einhorn H, Lawrence. Diagnosis and Treatment: Drugs Five Years Later - Cisplatin. Annals of Internal Medicine 1984; 100:704-713.

25. Comis RL. Cisplatin: The Future. Seminars in Oncology 1994; 21:109-113.

26. Sorenson CM, Eastman A. Influence of cis-Diamminedichloroplatinum(II)-induced on DNA synthesis and cell cycle progression in excision repair proficient and deficient chinese hamster ovary cells. Cancer Research 1988; 48:6703-6707.

27. Sorenson CM, Eastman A. Mechanism of cis-Diamminedichloroplatinum(II)-induced Cytotoxicity: Role of G2 Arrest and DNA Double-Strand Breaks. Cancer Research 1988; 48:4484-4488.

28. Sorenson CM, Barry MA, Eastman A. Analysis of Events Associated with Cell Cycle Arrest at G2 Phase and Cell Death Induced by Cisplatin. Journal of the National Cancer Institute 1990; 82:749-755.

29. Cece R, Barajon I, Tredici G. Cisplatin Induces Apoptosis in SH-SY5Y Human Neuroblastoma Cell Line. Anticancer Research 1995; 15:777-782.

30. Kondo S, Yin D, Morimura T, Kubo H, Nakatsu S, Takeuchi J. Combination therapy with cisplatin and nifedipine induces apoptosis in cisplatin-sensitive and cisplatin-resistant human glioblastoma cells. British Journal of Cancer 1995; 71:282-289.

31. Meyn RE, Stephens C, Hunter NR, Milas L. Kinetics of Cisplatin-induced Apoptosis in Murine Mammary and Ovarian Adenocarcinomas. International Journal of Cancer 1995; 60:725-729.

32. Chu G. Cellular Responses to Cisplatin. Journal of Biological Chemistry 1994; 269:787-790.

33. dE Graeff A, Slebos JC, Rodenhuis,S. Resistance to Cisplatin and analogues: Mechanisms And Potential Clinical Implications. Cancer Chemotherapy Pharmacology 1988; 22:325-332.

34. Andrews PA, Howell SB. Cellular Pharmacology Of Cisplatin: Perspectives On Mechanisms Of Acquired Resistance. Cancer Cells 1990; 2:35-43.

35. Tsujimoto Y, Finger LR, Yunis J, Nowell PC, Croce CM. Cloning of the chromosome breakpoint of neoplastic B cells with the t(14;18) chromosome translocation. Science 1984; 226:1097-1099.

36. Tsujimoto Y, Gorham J, Cossman J, Jaffe E, Croce CM. The t(14;18) chromosome translocation involved in B-cell neoplasms results from mistakes in VDJ joining. Science 1985; 229:1390

37. Cleary ML, Sklar J. Nucleotide sequence of a t(14;18) chromosomal breakpoint cluster region near a transcriptionally active locus on chromosome 18. Proceedings of the National Academy of Science USA 1985; 82:7439

38. Tsujimoto Y, Jaffe E, Croce CM. Involvement of the bcl-2 gene in human follicular lymphoma. Science 1985; 228:1440-1443.

39. Korsmeyer SJ. Bcl2: An Antidote to Programmed Cell Death. Cancer Surveys 1992; 15:105-118.

40. Tsujimoto Y, Croce CM. Analysis of the structure, transcripts, and protein products of bcl-2, the gene involved in human follicular lymphoma. Proceedings of the National Academy of Science 1986; 83:5214-5218.

41. Bakhshi A, Jensen JP, Goldman P, Wright JJ, McBride OW, Epstein AL. Cloning the chromosomal breakpoint of t(14;18) human lymphomas: clustering around J_H on chromosome 14 and near a transcriptional unit on 18. Cell 1985; 41:889-906.

42. Chen-Levy Z, Nourse J, Cleary ML. The *bcl-2* Candidate Proto-Oncogene Product is a 24-Kilodalton Integral-Membrane Protein Highly Expressed in Lymphoid Cell Lines and Lymphomas Carrying the t(14;18) Translocation. Molecular and Cellular Biology 1989; 9:701-710.

43. Hockenbery D, Nunez G, Milliman C, Schreibr RD, Korsmeyer SJ. Bcl-2 is an inner mitochondrial membrane protein that blocks programmed cell death. Nature 1990; 348:334-336.

44. Monaghan P, Robertson D, Andrew T, Amos S, Dyer MJS, Mason DY. Ultrastructural Localization of Bcl-2 Protein.. The Journal of Histochemistry and Cytochemistry 1992; 40:1819-1825.

45. Tsujimoto Y, Ikegaki N, Croce CM. Characterization of the protein product of bcl-2, the gene involved in human follicular lymphoma. Oncogene 1987; 2:3-7.

46. Tarduchy-Rodriguez G, Collins M, Lopez-Rivas A. Regulation of apoptosis in interleukin-3-dependent haemopoietic cells by interleukin-3 and calcium ionophores. EMBO Journal 1990; 9:2997-3002.

47. Vaux DL, Cory S, Adams JM. Bcl-2 gene promotes haemopoietic cell survival and cooperates with c-myc to immortalize pre-B cells. Nature 1988; 335:440

48. Nunez G, London L, Hockenbery DM, Alexander M, McKearn JP, Korsmeyer SJ. Deregulated Bcl-2 Gene Expression Selectively Prolongs Survival of Growth Factor-Deprived Haemopoietic Cell Lines. The Journal Of Immunology 1990; 144:3602-3610.

49. McDonnell TJ, Nunez G, Platt FM, Hockenbery DM, London L, McKearn JP. Deregulated Bcl-2-Immunoglobulin Transgene Expands a Resting but Responsive Immunoglobulin M and D-Expressing B-Cell Population. Molecular and Cellular Biology 1990; 10:1901-1907.

50. McDonnell TJ, Deane N, Platt FM. Bcl-2 Immunoglobulin transgenic mice demonstrate extended B cell survival and follicular lymphoproliferation. Cell 1989; 57:79-88.

51. Miyashita T, Reed JC. Bcl-2 Oncoprotein Blocks Chemotherapy-Induced Apoptosis in a Human Leukemia Cell Line. Blood 1993; 81:151-157.

52. Fisher TC, Milner AE, Gregory CD, Jackman AL, Aherne WG, Hartley JA. *bcl-2* Modulation of Apoptosis Induced by Anticancer Drugs: Resistance to Thymidylate Stress Is Independent of Classical Resistance Pathways. Cancer Research 1993; 53:3321-3326.

53. Sentman CL, Shutter JR, Hockenbery D, Kanagawa O, Korsmeyer SJ. *bcl-2* Inhibits Multiple Forms of Apoptosis but not Negative Selection in Thymocytes. Cell 1991; 67:879-888.

54. Sinha BK, Yamazaki H, Eliot HM, Schneider E, Borner MM, O'Connor PM. Relationships between proto-oncogene expression and

apoptosis induced by anticancer drugs in human prostate tumor cells. Biochimica et Biophysica Acta 1995; 1270:12-18.

55. Dole M, Nunez G, Merchant AK, Maybaum J, Rode CK, Bloch CA. Bcl-2 Inhibits Chemotherapy induced Apoptosis in Neuroblastoma. Cancer Research 1994; 54:3253-3259.

56. Hsu B, Marin M, Brisbay S, McConnell K, McDonnell T. Expression of bcl-2 gene confers multidrug resistance to chemotherapy-induced cell death. The Cancer Bulletin 1994; 46:125-129.

57. Oltvai ZN, Milliman C, Korsmeyer SJ. Bcl-2 Heterodimerizes In Vivo with a Conserved Homolog, Bax, That Accelerates Programmed Cell Death. Cell 1993; 74:609-619.

58. Boise LH, Gonzalez-Garcia M, Postema CE, Ding L, Lindstein T, Turka LA. *bcl-x,* a *bcl-2*-Related Gene That Functions as a Dominant Regulator of Apoptotic Cell Death.. Cell 1993; 74:597-608.

59. Sedlak TW, Oltvai ZN, Yang E, Wang K, Boise LH, Thompson CB. Multiple Bcl-2 family members demonstrate selective dimerizations with Bax. Proceedings of the National Academy of Science USA 1995; 92:7834-7838.

60. Sato T, Handa M, Bodrug S, Irie S, Iwama N, Boise LH. Interactions among members of the Bcl-2 protein family analyzed with a yeast two-hybrid system. Proceedings of the National Academy of Science USA 1994; 91:9238-9242.

61. Leighton J, Estes LW, Mansukhani S, Brada Z. A cell line derived from normal dog kidney (MDCK) exhibiting qualities of papillary adenocarcinoma and of renal tubular epithelium. Cancer 1970; 26:1022-1028.

62. Sandvig K, Van Deurs B. Toxin-induced Cell Lysis: Protection by 3-Methyladenine and Cycloheximide. Experimental Cell Research 1992; 200:253-262.

63. Allen J, Winterford C, Axelson RA, Gobe GC. Effects of hypoxia on morphological and biochemical characteristics of renal epithelial cell and tubule cultures. Renal Failure 1992; 14:453-460.

64. Flach R, Cattaruzza M, Koch-Brandt C. Clusterin gene expression in apoptotic MDCK cells is dependent on the apoptosis-inducing stimulus. Biochimica et Biophysica Acta 1995; 1268:325-328.

65. Hinshaw VS, Olsen CW, Dybdahl-Sissoko N, Evans D. Apoptosis: a Mechanism of Cell Killing by Influenza A and B Viruses. Journal of Virology 1994; 68:3667-3673.

66. Quinlan MP. E1A 12S in the absence of E1B or other co-operating oncogenes enables cells to overcome apoptosis. Oncogene 1993; 8:3289-3296.

67. Janiak F, Leber B, Andrews D, W. Assembly of Bcl-2 into Microsomal and Outer Mitochondrial Membranes. The Journal of Biological Chemistry 1994; 269:9842-9849.

68. Rago R, Mitchen J, Wilding G. DNA Fluorometric Assay in 96-Well Tissue Culture Plates Using Hoechst 33258 after Cell Lysis by Freezing in Distilled Water. Analytical Biochemistry 1990; 191:31-34.

69. Gavrieli Y, Sherman Y, Ben-Sasson SA. Identification of Programmed Cell Death In Situ via Specific Labelling of Nuclear DNA Fragmentation. The Journal of Cell Biology 1992; 119:493-501.

70. Thiry M. Highly Sensitive Immunodetection of DNA on Sections with Exogenous Terminal Deoxynucleotidyl Transferase and

Non-isotopic Nucleotide Analogues. The Journal of Histochemistry and Cytochemistry 1992; 40:411-419.

71. Mundle S, Iftikhar A, Shetty V, Dameron S, Wright-Quinones V, Marcus B. Novel In Situ Double Labelling for Simultaneous Detection of Proliferation and Apoptosis. The Journal of Histochemistry and Cytochemistry 1994; 42:1533-1537.

72. Gold R, Schmied M, Rothe G, Zischler H, Breitschope H, Wekerle H. Detection of DNA Fragmentation in Apoptosis: Application of In Situ Nick Translation to Cell Culture Systems and Tissue Sections. The Journal of Histochemistry and Cytochemistry 1993; 41:1023-1030.

73. Gorczyca W, Gong J, Darzynkiewicz Z. Detection of DNA Strand Breaks in Individual Apoptotic Cells by the in Situ Terminal Deoxynucleotidyl Transferase and Nick Translation Assays. Cancer Research 1993; 53:1945-1951.

74. Michelson AM, Orkin SH. Characterization of the Homopolymer Tailing Reaction Catalyzed by Terminal Deoxynucleotidyl Transferase. The Journal of Biological Chemistry 1982; 257:14773-14782.

75. Bollum FJ. Oligodeoxyribonucleotide-primed Reactions Catalyzed by Calf Thymus Polymerase. The Journal of Biological Chemistry 1962; 237:1945-1949.

76. Roychoudhry R, Jay E, Wu R. Terminal labelling and addition of homopolymer tracts to duplex DNA fragments by terminal deoxynucleotidyl transferase. Nucleic Acids Research 1976; 3:863-877.

77. Roychoudhry R, Wu R. Terminal Transferase-Catalyzed Addition of Nucleotides to the 3' Termini of DNA. Methods in Enzymology 1980; 65:43-62.

78. Nelson T, Brutlag D. Addition of Homopolymers to the 3'-Ends of Duplex DNA with Terminal Transferase. Methods in Enzymology 1979; 68:41-49.

79. Aull JL, Allen RL, Bapat AR, Daron HH, Friedman ME, Wilson JF. The Effects of Platinum Complexes on Seven Enzymes. Biochimica et Biophysica Acta 1979; 571:352-358.

80. Yi J, Michel O, Sassy-Prigent C, Chevalier J. Electron Microscopic Location of mRNA in the Rat Kidney: Improved Post-embedding In Situ Hybridization. The Journal of Histochemistry and Cytochemistry 1995; 43:801-809.

81. Fox KR, Higson SL, Scott JE. Methyl green and its analogues bind selectively to AT-rich regions of native DNA. European Journal of Histochemistry 1992; 36:263-270.

82. Seog K, Norden K, Norden B. A DNA major-groove binding drug. FEBS Letters 1993; 315:61-64.

83. Kurnick NB. Methyl Green-Pyronin. Journal of General Physiology 1949; 33:243-264.

84. Moffitt P. A Methyl Green-Pyronin Technique for Demonstrating Cell Death in the Murine Tumor S180. Cell Biology International 1994; 18:677-679.

85. Sinicropi D, Baker DL, Prince WS, Shiffer K, Shak S. Colorimetric Determination of DNase I activity with a DNA-Methyl Green Substrate. Analytical Biochemistry 1994; 222:351-358.

86. Bellon SF, Coleman JH, Lippard SJ. DNA Unwinding Produced by Site-Specific Intrastrand Cross-Links of the Antitumor Drug *cis*-Diamminedichloroplatinum (II). Biochemistry 1991; 30:8026-8035.

87. Gorczyca W, Bigman K, Mittleman A, Ahmed T, Gong J, Melamed MR. Induction of DNA Strand Breaks Associated with Apoptosis during Treatment of Leukemias. Leukemia 1993; 7:659-670.

88. Hickman J, Potten CS, Merritt AJ, Fisher TC. Apoptosis and cancer chemotherapy. Philosophical Transactions of the Royal Society of London - Series B:Biological Sciences 1994; 345:319-325.

89. Barry MA, Behnke CA, Eastman A. Activation of Programmed Cell Death (Apoptosis) by Cisplatin, Other Anticancer Drugs, Toxins and Hyperthermia. Biochemical Pharmacology 1990; 40:2353-2362.

90. Lasorella A, Iavarone A, Israel MA. Differentiation of Neuroblastoma Enhances Bcl-2 Expression and Induces Alterations of Apoptosis and Drug Resistance. Cancer Research 1995; 55:4711-4716.

91. Reed JC. Bcl-2: Prevention of Apoptosis as a Mechanism of Drug Resistance. Haematology/Oncology Clinics of North America 1995; 9:451-473.

92. Oltvai ZN, Korsmeyer SJ. Checkpoints of Duelling Dimers Foil Death Wishes. Cell 1994; 79:189-192.

93. Eastman A. Apoptosis: A Product of Programmed and Unprogrammed Cell Death. Toxicology and Applied Pharmacology 1993; 121:160-164.

94. Kulkarni GV, McCulloch CAG. Serum deprivation induces apoptotic cell death in a subset of Balb/c 3T3 fibroblasts. Journal of Cell Science 1994; 107:1169-1179.

95. Gerschenson LE, Rotello RJ. Apoptosis: a different type of cell death. FASEB J 1992; 6:2450-2455.

96. Lindenboim L, Haviv R, Stein R. Inhibition of Drug-Induced Apoptosis by Survival Factors in PC12 Cells. Journal of Neurochemistry 1995; 64:1054-1063.

97. Preston GA, Lang JE, Maronpot RR, Barrett CJ. Regulation of Apoptosis by Low Serum in Cells of Different Stages of Neoplastic Progression: Enhanced Susceptibility after Loss of a Senescence Gene and Decreased Susceptibility after Loss of a Tumor Suppressor Gene. Cancer Research 1995; 54:4214-4223.

98. Beck TW, Magnuson NS, Rapp UR. Growth factor regulation of cell cycle progression and cell fate determination. Current Topics in Microbiology and Immunology 1995; 194:291-303.

99. Taub M, Chuman L, Saier MH, Sato G. Growth of Madin-Darby canine kidney epithelial cell (MDCK) line in hormone-supplemented, serum-free medium. Proceedings of the National Academy of Science USA 1979; 76:3338-3342.

100. Estevez AG, Radi R, Barbeito L, Shin JT, Thompson JA, Beckman JS. Peroxynitrite-Induced Cytotoxicity in PC12 Cells:Evidence for an Apoptotic Mechanism Differentially Modulated by Neurotrophic Factors. Journal of Neurochemistry 1995; 65:1543-1550.

101. Frisch SM, Francis H. Disruption of Epithelial Cell-Matrix Interactions Induces Apoptosis. The Journal of Cell Biology 1994; 124:619-626.

102. Beere HM, Chresta CM, Alejo-Hergerg A, Skladanowski A, Dive CA, Larsen AK. Investigation of the Mechanism of Higher Order Chromatin Fragmentation Observed in Drug-Induced Apoptosis. Molecular Pharmacology 1995; 47:986-996.

103. Oberhammer F, Wilson JW, Dive CA, Morris ID, Hickman JA, Wakeling AE. Apoptotic death in epithelial cells: cleavage of DNA to 300 and/or 50 kb fragments prior to or in the absence of internucleosomal fragmentation. EMBO Journal 1993; 12:3679-3684.

104. Peitsch MC, Mannherz HG, Tschopp J. The apoptosis endonucleases: cleaning up after cell death? Trends in Cell Biology 1994; 4:37-41.

105. Jarvis DW, Kolesnick RN, Fornari FA, Traylor RS, Gewirtz DA, Grant S. Induction of apoptotic DNA damage and cell death by activation of the sphingomyelin pathway. Proceedings of the National Academy of Science USA 1994; 91:73-77.

106. Mighell A, Cavalla P, Marino S, Schiffer D. A Study of Apoptosis in Normal and Pathologic Nervous Tissue After in situ End-Labelling of DNA Strand Breaks. Journal of Neuropathology and Experimental Neurology 1994; 53:606-616.

107. Ansari B, Coates PJ, Greenstein BD, Hall PA. In Situ End-Labelling Detects DNA Strand Breaks in Apoptosis and other Physiological and Pathological States. Journal of Pathology 1993; 170:1-8.

108. Migheli A, Attanasio A, Schiffer D. Ultrastructural Detection of DNA Strand Breaks in Apoptotic Neural Cells by *in situ* end-labelling Techniques. Journal of Pathology 1995; 176:27-35.

109. Eastman A, Barry M, A. The Origins of DNA Breaks: A Consequence of DNA Damage, DNA Repair or Apoptosis? Cancer Investigation 1992; 10:229-240.

110. Coates PJ, Save V, Ansari B, Hall PA. Demonstration of DNA Damage/Repair in Individual Cells Using *In Situ* End Labelling:

Association of p53 with Sites of DNA Damage. Journal of Pathology 1995; 176:19-26.

111. Wijsman JH, Jonker RR, Keijzer R, Van De Velde CJH, Cornelisse CJ, Hein Van Dierendonck J. A New Method to Detect Apoptosis in Paraffin Sections: In Situ End-Labelling of Fragmented DNA. The Journal of Histochemistry and Cytochemistry 1993; 41:7-12.

112. Kraupp-Grasl B, Ruttkay-Nedecky B, Koudelka H, Bukowska K, Bursch W, Schulte-Hermann R. In Situ Detection of Fragmented DNA (TUNEL Assay) Fails to Discriminate Among Apoptosis, Necrosis and Autolytic Cell Death: A Cautionary Note. Hepatology 1995; 21:1465-1468.

113. Gold R, Schmied M, Giegerich G, Breitschopf H, Hartung HP, Toyka KV. The Two In Situ Techniques do not Differentiate between Apoptosis and Necrosis but rather Reveal Distinct Patterns of DNA Fragmentation in Apoptosis. Laboratory Investigation 1995; 72:611-613.

114. Bompart G, Orfila C, Girolami J. Distal nephrotoxicity of cisplatin demonstrated by urinary kallikrein excretion and morphological study in rats. Toxicology 1991; 69:121-132.

115. Aggarwal SK. A Histochemical Approach to the Mechanism of Action of Cisplatin and Its Analogues. The Journal of Histochemistry and Cytochemistry 1993; 41:1053-1073.

116. Goldstein RS, Mayor GH. The Nephrotoxicity Of Cisplatin. Life Sciences 1983; 32:685-690.

117. Choie DD, Del Campo AA, Guarino AM. Subcellular Localization of *cis*-Dichlorodiammineplatinum (II) in Rat Kidney and Liver. Toxicology and Applied Pharmacology 1980; 55:245-252.

118. Hartshorn EA. Drug Interactions and Reactions Update: Newer Insights into Cisplatin Nephrotoxicity. The Annals of Pharmacology 1993; 27:1519-1525.

119. Kataoka A, Kubota M, Wakazono Y, Okuda A, Bessho R, Wei Lin Y. Association of high molecular weight DNA fragmentation with apoptotic or non-apoptotic cell death induced by calcium ionophore. FEBS Letters 1995; 364:264-267.

120. Fairbairn LJ, Cowling GJ, Dexter M, Rafferty JA, Margison GP. *bcl-2* Delay of Alkylating Agent-Induced Apoptotic Death in a Murine Haemopoietic Stem Cell Line. Molecular Carcinogenesis 1994; 11:49-55.

121. Catchpoole DR, Stewart BW. Formation of Apoptotic Bodies is Associated with Internucleosomal DNA Fragmentation during Drug-Induced Apoptosis. Experimental Cell Research 1995; 216:169-177.