P53 AND DNA DAMAGE-INDUCED APOPTOSIS IN OVARIAN TUMOUR CELL LINES

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ABSTRACT

The A2780 human ovarian adenocarcinoma cell line was used to establish methodologies for the detection and quantitation of ionising radiation- and cisplatin-induced apoptosis. Detection of apoptosis in A2780 cells by field inversion gel electrophoresis of DNA was found to be reliable and accurate. Apoptosis was quantitated using fluorescent end-labelling of DNA fragments by TUNEL/PI staining followed by FACs analysis. Cells displaying apoptotic morphology (dense chromatin, pyknotic nuclei around the cell margin, apoptotic bodies) when viewed by confocal microscopy were found to be those which also displayed increased green fluorescence due to TUNEL staining.

A2780 cells engaged apoptosis in response to DNA damage generated by both ionising radiation and cisplatin. The DNA of apoptotic A2780 cells degraded in a non-random manner to produce DNA fragments of two sizes: one of 50kbp and one of 300-1000kbp. The DNA did not degrade further to produce internucleosommal fragments. Maximal apoptosis occurred at either 72h or 96h after both cisplatin and radiation.

Ionising radiation-induced apoptosis was reduced in the A2780/cp70 cell line compared to A2780 cells. Cisplatin-induced apoptosis was reduced in nine out of ten cisplatin resistant cell lines derived *in vitro* from A2780 cells. A higher level of Bcl-2 was observed in A2780/cp70 and this may contribute to the lack of apoptosis seen in these cells.

The role of p53 in ionising radiation- and cisplatin-induced apoptosis in A2780 cells was investigated using a dominant negative mutant *TP53* (codon 143, val to ala)-transfected cell line, A2780/mp53. A2780/mp53 cells underwent less apoptosis than the vector-transfected control cell line after equal doses of cisplatin or ionising radiation.

These results showed that DNA damage caused by cisplatin or ionising radiation resulted in cell death by apoptosis in A2780 cells which was reduced in resistant cell lines. In addition they indicate a role for functional p53 in the ionising radiation- and cisplatin-induced apoptotic responses of A2780 cells.

ABBREVIATIONS

ala	alanine
APS	ammonium persulphate
bp	base pair
b-	biotinylated-
BSA	bovine serum albumin
cDNA	complementary deoxyribonucleic acid
cm	centimetre
°C	degrees Centigrade
dCTP	deoxycytosine triphosphate
dUTP	deoxyuridine triphosphate
ddH_2O	double distilled water
DTT	di-thiothreitol
DMSO	dimethyl sulphoxide
DNA	deoxyribonucleic acid
EDTA	ethylenediaminetetraacetic acid
FACs	fluorescence-assisted cell sorting
FIGE	field inversion gel electrophoresis
FITC	fluorescein isothiocyanate
g	gram
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
Gy	Gray
h	hour
HRP	horse radish peroxidase
Ig	immunoglobulin
kbp	kilobase pair
kDa	kiloDalton
M	molar
mA	milliamp
min.	minute
ml	millilitre
µl	microlitre
mm	millimetre
mM	millimolar
µM	micromolar
mg	milligram
µg	microgram
nm	nanomole
NP-40	Nonidet P-40
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate-buffered saline
PE	phosphate-buffered EDTA
PI	propidium iodide
PIPES	1,4-piperazinediethane-sulphonic acid
PMSF	phenylmethylsulphonyl fluoride
rpm	revolutions per minute
s	second
SDS	sodium dodecyl sulphate
SF	surviving fraction
SSC	saline-sodium citrate
TdT	terminal DNA transferase
TEMED	$N_1N_1N_1N_1$-tetramethyl-ethylene diamine
TRIS	2-amino-2-hydroxymethyl-1,3-propanediol-hydrochloride
TUNEL	terminal DNA transferase-mediated b-dUTP nick end labelling
UV	ultra-violet
V	Volt
val	valine

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INTRODUCTION

1.1 Apoptosis

Apoptosis is a morphological phenomenon that occurs as a result of a cell engaging a programmed cell death pathway following growth factor deprivation or treatment with cytotoxic agents. It occurs as the result of exposure to a wide variety of DNA damaging agents, including cisplatin and ionising radiation, in many different cell types (reviewed in Kerr et al., 1994). Apoptosis had been observed as early as 1885 when it was referred to as chromatolysis (reviewed in Majno and Joris, 1995) but it's importance was not recognised until the early 1970's when it's possible role in the regulation of cell populations was realised (Kerr et al., 1972; Kerr, 1971).

Apoptosis is under genetic control (Wyllie, 1994) and is a metabolically active process. Morphologically, the cell shrinks and becomes denser. The chromatin becomes pyknotic and packed into smooth masses around the cell margin. The nucleus then breaks up by producing processes containing fragments of chromatin. These processes break off to form apoptotic bodies which may be phagocytised by macrophages or neighbouring cells (Kerr and Harmon, 1991; Majno and Joris, 1995). Biochemically, the DNA is broken down into either large fragments of 50kbp and/or 300-1000kbp (Oberhammer et al., 1993) or into smaller 180-200bp internucleosomal fragments known as the DNA ladder (Wyllie, 1980). Apoptotic morphology may also be observed in the absence of DNA fragmentation (Sun et al., 1994; Cohen et al., 1992).

1.2 The apoptotic signalling cascade

Apoptosis is the morphological manifestation of a programmed cell death cascade which can be divided into several stages. Multiple signalling pathways lead from death-inducing extracellular agents to a control and execution stage which triggers the onset of

morphological changes. Extensive work has been done to elucidate the roles of the many proteins which appear to be involved in the control and execution of apoptosis. The mammalian *BCL-2* gene product is an inhibitor of apoptosis (Reed et al., 1995) and is homologous to the *ced-9* gene of *Caenorhabditis elegans* (*C. elegans*) (Hengartner and Horvitz, 1994; Hengartner et al., 1992). The *ced-9* gene regulates *ced-3* which promotes programmed cell death during *C. elegans* development (Ellis and Horvitz, 1986). The *ced-3* gene product is homologous to the mammalian ICE-like (interleukin 1β converting enzyme-like) cysteine protease which is a promoter of apoptosis (Miura et al., 1993).

Overexpression of ICE in mammalian cells induces apoptosis which can be inhibited by Bcl-2 (Miura et al., 1993). Bcl-2 is a member of a large family of related proteins which regulate apoptosis by forming both homodimers and heterodimers (White, 1996; Oltvai et al., 1993 and references therein). These include promoters of apoptosis: Bcl-x_S, Bax, Bad, Bik1 and Bak (which can also inhibit apoptosis), and inhibitors of apoptosis: Bcl-2, Bcl-x_L, Mcl-1, and E1B 19K. ICE is a member of a large family of related highly specific proteases known as caspases reviewed in (Martins and Earnshaw, 1997). Cisplatin can induce the expression of ICE in murine and human glioma cells during apoptosis (Kondo et al., 1995). Cisplatin-induced apoptosis and ICE overexpression can be suppressed by Bcl-2 and Bcl-x_L in this model suggesting a role for the ICE/Bcl-2 signalling cascade in cisplatin-induced apoptosis. The apoptosis observed in this case was independent of the p53 status of the cell lines. Ionising radiation-induced apoptosis has been found to be associated with increased expression of Bcl-x_L (Zhan et al., 1996). The induction of Bcl-x_L by ionising radiation was dependent on normal p53 function. It would appear that the ICE/Bcl-2 cascade can be induced by both ionising radiation and cisplatin and can trigger apoptosis in either p53-functional or -dysfunctional cell types. In addition

p53 can down-regulate the expression of Bcl-2 and up-regulate the expression of Bax in murine leukemia cells (Miyashita et al., 1994) and is a direct transciptional activator of the human *BAX* gene (Miyashita and Reed, 1995).

1.3 The DNA-damage response

1.3.1 Recognition of damage

In order to repair damage introduced into DNA by agents such as cisplatin the lethal adducts must first be identified. This can then facilitate the initiation of a signalling pathway which may ultimately lead either to repair and survival, to cell death or to tolerance of the damage.

Mammalian proteins have been identified, which can bind to DNA damaged by various agents including cisplatin. Human single-stranded DNA binding protein (HSSB, now known as RPA) which is involved in mammalian excision repair, binds to DNA treated *in vitro* with cisplatin (Clugston et al., 1992). DNA-cisplatin adducts are also recognised and bound by a high mobility group protein, HMG1 (Pil and Lippard, 1992) and by SSRP1 (Bruhn et al., 1992). The role of these damage recognition proteins (DRP's) in resistance to cisplatin has been investigated. A higher level of DRP's was seen in a cisplatin-resistant human ovarian tumour cell line compared to the sensitive parental cell line (McLaughlin et al., 1993). However there was no difference in the binding activity in extracts from tumours of different cisplatin sensitivity (Bissett et al., 1993).

It has been suggested that signalling pathways are induced following ionising radiation damage due to the relaxation of DNA supercoiling, triggered by the DNA single strand breaks (Povirk and Painter, 1976). Many genes induced in response to the

oxidative stress of ionising radiation have been identified, including transcription factors, proteases, protease inhibitors and proteins affecting cell proliferation (reviewed in Janssen et al., 1993). One of these, the transcription factor p53 is known to accumulate following ionising radiation (or cisplatin) treatment of cells *in vitro* (Brown et al., 1993; Kastan et al., 1991; Maltzman and Czyzyk, 1984). The C-terminus of the p53 protein has also been shown to bind with increased affinity to DNA damaged by ionising radiation (Reed et al., 1995). The role of p53 in the DNA-damage response pathway is discussed in greater depth in the following sections.

1.3.2 The role of p53 in the DNA damage response pathway

1.3.2.1The p53 tumour suppressor protein

The human *TP53* gene is approximately 20kb in size and contains 11 exons (Lamb and Crawford, 1986). The product of this gene is a protein which contains five evolutionarily conserved regions, the first of which is proximal to the amino terminus (amino acid residues 13-19) and the other four are clustered centrally, between residues 100 and 300 (Soussi et al., 1990). These are the DNA binding domains (Cho et al., 1994). The C-terminus contains the tetramerisation domain (Sturzbecher et al., 1992) and nuclear localisation signals which are essential for p53 activity (Shaulsky et al., 1991). Residues 1-43 of the N-terminus contain the transactivation domain (Unger et al., 1992).

The p53 protein is a nuclear phosphoprotein (M_r 53,000) of 393 amino acids that acts as a tumour suppressor protein. It was discovered as a protein bound to the SV40 large T antigen (Lane and Crawford, 1979). The p53 protein can bind DNA at certain specific contact points on the protein and is capable of sequence specific binding

(Kern et al., 1991) which is associated with growth suppression (Pietenpol et al., 1994). It is a transcriptional transactivator which can regulate various genes including *WAF1/CIP1* (Harper et al., 1993; El-Deiry et al., 1993), *CYCLIN G* (Okamoto and Beach, 1994) and *MDM2* (Wu, 1993; Oliner et al., 1993; Momand et al., 1992). The wild-type p53 protein is capable of suppressing the tumorigenic phenotype in a variety of cell types (Johnson et al., 1991; Casey et al., 1991; Baker et al., 1990; Finlay et al., 1989). Certain mutant forms of *TP53*, if transfected into normal cells containing wild-type p53, cause cellular transformation (Hinds et al., 1989; Eliyahu et al., 1988) and the endogenous wild-type p53 can be driven into a mutant conformation (Milner and Medcalf, 1991). This causes abrogation of wild-type p53 binding and transactivation activities (Kern et al., 1992; Farmer et al., 1992). As mentioned above, p53 accumulates in response to ionising radiation and other DNA-damaging agents. This accumulation may occur by a post-translational mechanism (Kastan et al., 1991) possibly as a result of an increased half-life of the protein leading to enhanced stability. Alternatively it has been found that the p53 3'UTR (untranslated region) is involved in translational repression which is alleviated following DNA damage by γ-irradiation (Fu and Benchimol, 1997).

1.3.2.2 Mutations in *TP53*

Missense mutation in one allele of *TP53* with concomitant loss of heterozygosity of the other allele is the most common genetic alteration in human cancer (Hollstein et al., 1991; Takahashi et al., 1989; Nigro et al., 1989). Eighty to ninety percent of mutations in the *TP53* gene are missense mutations leading to a conformational change in the protein (Greenblatt et al., 1994). The *TP53* gene is one of the most frequently encountered genetic alterations in solid tumours (Toma et al., 1992) and is found to be mutated in 25-80% of ovarian tumours (Teneriello et al., 1993; Milner

et al., 1993; Kupryjanczyk et al., 1993; Kohler et al., 1993; Okamoto et al., 1991; Mazars et al., 1991; Marks et al., 1991). The majority of mutations (80-90%) are found between codons 100 and 300 which spans the four centrally clustered conserved domains (Soussi et al., 1990).

1.3.2.3 p53 and cell cycle arrest

Following routine damage of DNA, cell cycle arrests are thought to be implemented in order to allow time for repair before DNA replication (G1- and S-phase arrests) or segregation of chromosomes (G2-phase arrest). The p53 protein is a crucial player in the G1/S cell cycle checkpoint. Following γ-irradiation of wild-type p53 cells there is a transient induction of the p53 protein which correlates with the G1 arrest (Kastan et al., 1991). It was found that p53 could transcriptionally activate the *WAF1/CIP1* gene (El-Deiry et al., 1993) whose gene product, $p21^{WAF1/CIP1}$ could inhibit the G1 cyclin-dependent kinases (Harper et al., 1993). The $p21^{WAF1/CIP1}$ protein is induced by exposure to DNA-damaging agents in wild-type p53-containing cells that are undergoing p53-dependent G1 arrest (El-Deiry et al., 1994). The ionising radiation-induced G1 arrest is completely abrogated in $p21^{WAF1/CIP1}$-deficient cells (Waldman et al., 1995) demonstrating the absolute necessity for $p21^{WAF1/CIP1}$ induction in this DNA damage response. In addition, neither p53 nor $p21^{WAF1/CIP1}$ are required for the ionising radiation-induced G2 cell cycle arrest (Levedakou et al., 1995; McIlwrath et al., 1994) demonstrating the specificity for the G1 arrest in this DNA damage response pathway. The $p21^{WAF1/CIP1}$ protein forms a complex with PCNA and GADD45 thereby exerting its effects on the G1 cyclin-dependent kinases (Smith et al., 1994; Waga et al., 1994). Aternatively the $p21^{WAF1/CIP1}$ protein may be able to inhibit G1-S phase transition by directly binding the G1 cyclin-dependant kinases (Ogryzko et al., 1997). The $p21^{WAF1/CIP1}$

protein was in fact found to be a universal inhibitor of cyclin-dependent kinases (Xiong et al., 1993) however only the $p21^{WAF1/CIP1}$-induced G1 arrest is dependent on wild-type p53 (Lin et al., 1992; Kuerbitz et al., 1992; Kastan et al., 1991).

1.3.2.4 p53 and apoptosis

When a cell is exposed to a DNA damaging agent it can arrest in either G1/S or G2, presumably to allow the cell time to repair the damage. Alternatively, the DNA damaging agent may trigger the cell to enter a cell death pathway. Wild-type p53 is required for ionising radiation-induced apoptosis in mouse thymocytes (Clarke et al., 1993; Lowe et al., 1993). There is growing evidence that this induction of p53-dependent apoptosis occurs by a different pathway to the p53-dependent G1/S cell cycle arrest and does not require the induction of $p21^{WAF1/CIP1}$ (Friedlander et al., 1996; Ludwig et al., 1996; Attardi et al., 1996; Rowan et al., 1996; Haupt et al., 1995). There is conflicting evidence for the requirement of p53 transcriptional activation of target genes for apoptosis. In some cases it has been found that p53 transcriptional activation is dispensable for the induction of apoptosis (Haupt et al., 1995; Wagner et al., 1994; Caelles et al., 1994) while on the other hand, specific defects in transcriptional activation have been found to correlate with impaired apoptotic function (Ludwig et al., 1996). It would seem that the requirement for p53 transcriptional activation is highly dependent on the genetic context and on the apoptotic effector.

1.3.2.5 Loss of p53 function and resistance in ovarian carcinoma cells

Human ovarian adenocarcinoma cells which have been transfected *in vitro* with a mutant *TP53* (codon 143, val-ala) lose wild-type p53 function as measured by a loss of the DNA damage-induced G1 cell cycle arrest (Brown et al., 1993). These mutant p53-containing cells become more resistant to ionising radiation (McIlwrath et al., 1994) and to a variety of other cytotoxic agents (Vasey et al., 1996) as compared with the intrinsically sensitive parental cells and cells transfected with vector alone. In addition, p53 function was found to correlate with intrinsic radiosensitivity in a panel of cell lines which reflected the radiosensitivity of the tumours from which they were derived (McIlwrath et al., 1994). Cells selected in vitro for cisplatin resistance have been shown to have increased levels of p53 protein and are dysfunctional for DNA damage-induced G1 arrest (Brown et al., 1993). In addition they are cross resistant to ionising radiation and multiple chemotherapeutic agents (Vasey et al., 1996; Vasey et al., 1995).

1.4 Cisplatin

1.4.1 Modes of cytotoxicity

Cisplatin (or *cis*-dichlorodiammineplatinum(II)) is widely used in the chemotherapeutic treatment of ovarian carcinoma (as well as in testicular and head and neck carcinoma). Ovarian cancer is the fifth most common type of cancer among females in the UK. Treatment of ovarian carcinoma typically involves surgical resection, followed by chemotherapy and/or local radiation. Cisplatin's cytotoxic effects against cells in culture are directly related to total platinum binding to DNA (Knox et al., 1986), to cisplatin-DNA interstrand crosslinks (Zwelling et al., 1979) and to intrastrand crosslinks

at the N7 position of adjacent guanine bases (Eastman, 1987; Eastman, 1983). This latter adduct is thought to be the main mechanism by which cisplatin exerts it's cytotoxicity.

1.4.2 Cellular responses to cisplatin

Treatment of cells *in vitro* with cisplatin causes cell cycle perturbations (G1/S and G2 phase cell cycle arrests) and cell death by apoptosis (Ormerod et al., 1994; Demarcq et al., 1992). Cisplatin can induce an increase in accumulation of wild-type p53 protein in ovarian tumour cells (Brown et al., 1993) and can thereby lead to increased mRNA levels of p53 activated genes such as *WAF1/CIP1* (El-Deiry et al., 1993).

1.4.3 Mechanisms of cisplatin resistance

As with most cancer therapies the recurrence of disease is a major consideration. Initially sensitive tumours, such as ovarian carcinoma can acquire multidrug resistance after exposure to a single agent (Kaye, 1988). This phenomenon is also observed *in vitro* after multiple (Vasey et al., 1996; Hamaguchi et al., 1993; Behrens et al., 1987) or single (McLaughlin et al., 1991) exposures to cisplatin. Possible mechanisms of acquired cisplatin resistance which may not involve p53 include decreased cisplatin uptake and accumulation (reviewed in Gately and Howell, 1993) and increases in glutathione or metallothionein levels in resistant cells (reviewed in Andrews and Howell, 1990). A genetic mechanism for decreased cisplatin uptake has not been elucidated and in no case is the change greater than 50% suggesting that this theory could not fully explain acquired cisplatin resistance. Increases in glutathione and metallothionein levels have only been observed in cells selected for resistance by chronic exposure to cisplatin (Andrews et al., 1990). When 2-3-fold cisplatin-resistant cells were selected from the

human ovarian carcinoma cell line, 2008, no elevation in glutathione level was observed (Andrews et al., 1985). In addition, an increase in metallothionein was not observed in cell lines obtained from patients' tumours that were refractory to cisplatin (Schilder et al., 1990). Other mechanisms which may contribute to acquired cisplatin resistance include adduct tolerance, increased recognition or repair of cisplatin adducts (see below) or loss of some component of the cell death signalling pathway.

1.4.4 DNA repair and cisplatin resistance

Nucleotide excision repair is thought to be the main mechanism by which cells remove cisplatin adducts from DNA (Hansson and Wood, 1989; Sibghat-Ullah et al., 1989). The lines of evidence for differences in adduct removal being the cause of resistance however, are contradictory. Some investigators have observed an increase in adduct removal in cisplatin-resistant cells compared to their cisplatin-sensitive parental lines (Masuda et al., 1990; Eastman and Schulte, 1988). Others have found no correlation between cisplatin sensitivity and adduct removal (Shellard et al., 1991; Terheggen et al., 1990; Bedford et al., 1988). In addition, in cases where increased adduct removal has been seen, the cells are 10- to 100-fold more resistant than the parental lines and increases in adduct removal of 3- to 4-fold would not seem to correlate with the high levels of resistance. It would seem that differences in DNA repair are dependent on both the cell type under investigation and the mode of selection for cisplatin resistance. It has been suggested that cisplatin resistance may be due to tolerance of cisplatin-DNA adducts and indeed cisplatin-resistant A2780 cells can tolerate four times as much cisplatin on their DNA as sensitive cells at equitoxic concentrations (Johnson et al., 1994b; Schmidt and Chaney, 1993). One possible mechanism of tolerance is by adduct bypass (Mamenta et al., 1994). Another possible

mechanism is the selective repair of transcriptionally active genes (Zhen et al., 1992). Tolerance of resistant cells to cisplatin adducts is concordant with the cells having a defect in the signalling pathway that leads to cell death.

1.5 Ionising radiation

1.5.1 Modes of cytotoxicity

Ionising radiation has been used as an adjuvant to surgery, as treatment for non-resectable disease or as palliation for advanced ovarian cancer. Postoperative irradiation has also been used in cancer therapy. Exposure of cells to ionising radiation results in a variety of DNA lesions via the action of free radicals which cause oxidative damage. These lesions include DNA base alterations, DNA-DNA and DNA-protein cross-links, and single- and double-strand DNA breaks (Ward, 1997). Of these, the double strand break is the primary lethal lesion. Double-strand DNA breaks can lead to lethal chromosomal aberrations (Phillips and Morgan, 1992) and mammalian cells with deficient or delayed double-strand break repair are more sensitive to ionising radiation (Jeggo, 1990). There is also some evidence from experiments performed on bovine aorta endothelial cells that radiation can induce cell death through hydrolysis reactions in the plasma membrane (Haimovitz-Friedman et al., 1994).

Apart from the clinical relevance of studying the response of cells to radiation and the acquisition of radio-resistance *in vitro,* ionising radiation also presents a means of introducing lethal lesions into DNA in the form of double strand DNA breaks , the cellular reaction to which may be considered without the complicating factor of the pharmacokinetics of chemotherapeutic drugs.

1.5.2 Radiation resistance

Acquired radioresistance can occur in some clinical situations (Miller et al., 1992) and following selection of cells *in vitro* for resistance to cisplatin (Britten et al., 1992; Behrens et al., 1987). The mechanisms by which tumorigenesis confers resistance to ionising radiation (McKenna et al., 1990) are thought to include changes in damage processing and altered cellular metabolism (reviewed in Powell and Abraham, 1993). These mechanisms may be common to those involved in acquired cisplatin and multi-agent drug resistance.

1.6 Project aims

The aims of the project were:

(i) to determine whether or not cisplatin and ionising radiation are capable of inducing apoptosis in ovarian tumour cell lines expressing wild-type p53

(ii) to determine whether or not the apoptosis induced by these agents is dependent on p53 in these cells

(iii) to determine whether or not the acquisition *in vitro*, of cisplatin resistance (and cross resistance to ionising radiation) is due to a loss of, or reduction in the apoptotic response.

CHAPTER 2

MATERIALS AND METHODS

2.1 Materials

2.1.1 Chemicals

Chemicals were obtained from the following companies except where indicated in the text:

BDH Chemicals Ltd, Supplied by McQuilken & Co, Glasgow, UK

Gibco BRL, Paisley, Renfrewshire, UK

Pharmacia Biotech, Milton Keynes, Bucks, UK

Sigma Chemicals, Poole, Dorset, UK

Boehringer Mannheim, Lewes, East Sussex, UK

2.1.2 Radiochemicals

(α^{32}P)dCTP for random labelling of DNA for Southern hybridisation was obtained from Amersham International plc, Little Chalfont, Bucks, UK.

2.1.3 Equipment

Routine equipment which would be an integral part of any laboratory is not listed.

Flow Cytometer	Becton Dickinson, Oxford, UK
Milliblot SDE Electroblotter	Millipore, Watford, UK
X-Ray Developer	Kodak, Cambridge, UK
UV Crosslinker	Stratagene, Cambridge, UK
Hybridisation Oven	Hybaid Ltd, Middlesex, UK
Electrophoresis Tanks	Biorad Labs Ltd, Watford, UK; IBI Ltd, Cambridge, UK

Luminescence Spectrometer	
LS50	Perkin Elmer, Beaconsfield, Bucks.,UK
UV Transilluminator and	
Digital Imaging System	Appligene, Chester-Le-Street, Co.Durham,UK

2.1.4 Enzymes

Terminal DNA transferase was bought from Boehringer Mannheim. Proteinase K was supplied by Gibco BRL.

2.1.5 Size markers

High range pre-stained molecular weight markers (Gibco BRL) were used for SDS-PAGE gels.

HindIII digested phage λ (IBI, Cambridge, UK) and 100bp DNA ladder (Gibco BRL) were used for normal agarose gels.

Full length Lambda DNA (Boehringer Mannheim) and Lambda ladder (Promega, Southampton, UK) were used for FIGE gels.

2.1.6 Buffers and solutions

All solutions were made with double-distilled water (ddH_2O) and stored at room temperature, except where indicated.

Streptavidin-FITC solution

4 x SSC

0.1% Triton X-100

5μg/ml fluoresceinated-avidin

5% Marvel (non-fat dry milk)

(prepared immediately prior to use)

Blocking solution (for Westerns)

1 x Blotto

5% Marvel

0.01% v/v Tween-20

1mM DTT

Blotto(10x)

500mM Tris pH7.5

500mM NaCl

10mM EDTA

Cacodylate buffer

200mM Potassium cacodylate

2.5mM Tris.HCl (pH6.6)

2.5mM $CoCl_2$

0.25mg/ml BSA

(prepared immediately prior to use)

Denaturation buffer

0.5M NaOH

1.5M NaCl

DNA loading buffer

30% w/v Glycerol

0.25% w/v Bromophenol blue

0.25% w/v Xylene cyanol

Genescreen

0.5M Na_2HPO_4

0.5M NaH_2PO_4

High salt lysis buffer

500mM NaCl

1% NP-40

50mM Tris pH7.5

1mM DTT (added fresh)

Protease inhibitors (1x, added fresh)

(Stored at 4°C)

Hoechst 33258-TNE

10mM Tris pH7.6

5mM NaCl

2mM EDTA

0.1μg/ml Hoechst 33258 (added immediately prior to use)

Hybridisation buffer

50mM PIPES (pH6.8)

50mM Na_2HPO_4

50mM NaH_2PO_4

100mM NaCl

1mM EDTA

5% SDS

Lysis buffer (for DNA from whole cells)

100mM Tris.HCl (pH8.5)

5mM EDTA (pH8)

0.2% SDS

200mM NaCl

100μg/ml Proteinase K (added immediately prior to use)

L buffer (for DNA from agarose pellets)

100mM Tris.HCl (pH7.6)

100mM EDTA (pH8)

1% SDS

20mM NaCl

1mg/ml Proteinase K (added immediately prior to use)

Neutralisation buffer

1M Tris (pH7.4)

1.5M NaCl

Protease inhibitors (100x)

0.1mg/ml aprotinin

0.1mg/ml pepstatin

0.1mg/ml chymostatin

0.05M benzamidine

0.05M PMSF

0.1mg/ml leupeptin

(all stored at -20°C)

5 x Protein loading buffer

250mM Tris (pH8)

10% SDS

10mM EDTA

50% Glycerol

0.25% w/v Bromophenol blue

0.25% w/v Xylene cyanol

Phosphate buffered saline (PBS)

0.8% NaCl

0.115% Na_2HPO_4

0.02% KCl

0.02% KH_2PO_4

PBS-T

PBS

0.1% Tween-20

0.5% BSA fraction V (w/v)

(Stored at 4°C)

Running gel buffer

1.5M Tris (pH8.9)

4% SDS

Spacer gel buffer

0.5M Tris (pH6.7)

0.4% SDS

20 x SSC (pH7)

3M NaCl

300mM tri-Sodium citrate

TAE(1x)

40mM Tris (pH8)

2mM EDTA

20mM NaCl

20mM Na Acetate

Tank buffer (5x)

250M Tris

4% Glycine

0.5 SDS

TBE(1x) pH8

89mM Tris borate

89mM Boric acid

2.5mM EDTA

TE pH8

10mM Tris

1mM EDTA

Transfer buffer

48mM Tris

39mM Glycine

0.037% v/v SDS

20% Methanol

Wash buffer

1 x SSC

5% SDS

2.1.7 Cell lines

A2780	A human, ovarian adenocarcinoma cell line obtained from R.F. Ozols and T.C. Hamilton, Fox Chase Cancer Center, Philadelphia.
A2780cp70	A cisplatin resistant derivative of A2780 isolated after exposure to the drug for two years.

For a more complete description of A2780 and A2780cp70 see Behrens et al., (1987).

A2780/mp53	A2780 clonal cell line transfected with the plasmid pC53-SCX3 containing mutant *TP53* cDNA (codon 143, val to ala) expressed from a CMV promoter (Brown et al., 1993).
A2780/v	A2780 clonal cell line transfected with the pC53-SCX3 plasmid (vector alone).
A2780/mcp1 through 9	Independent, multiple step-selected cisplatin resistant derivatives of A2780 isolated by exposure to increasing concentrations of the drug from 1μM to 15μM (Anthoney et al., 1996).

2.1.8 Antibodies and immunological reagents

Mouse monoclonal anti-p53 antibody (Ab-6) was purchased from Oncogene Science, Cambridge, UK and was used at a concentration of 0.1μg/ml.

Mouse monoclonal anti-Bcl-2 antibody was purchased from DAKO (High Wycombe, UK) and was used at a concentration of 4μg/ml.

Anti-mouse Ig horseradish peroxidase conjugate (from sheep) was obtained from Amersham International and was used at a 1 : 5000 dilution.

2.2 Methods

2.2.1 Preparation of DNA

2.2.1.1 Extraction of DNA for agarose gel electrophoresis

Genomic DNA was prepared from cell monolayers by direct addition of lysis buffer followed by isopropanol precipitation, according to Laird et al., (1991).

2.2.1.2 Preparation of DNA for field inversion gel electrophoresis

DNA was prepared for field inversion gel electrophoresis (FIGE) according to Sambrook et al., (1989). Cells were seeded into $10cm^2$ plates at $2.5x10^3$cells/plate and allowed to grow for 3 days. The medium was changed for fresh medium before irradiation and at various times after irradiation cells were harvested by trypsinisation and resuspended in ice cold PBS at a concentration of $4x10^6$cells/100µl PBS. After warming the cell suspension to 42°C an equal volume of 2% low gelling temperature agarose at 42°C was added to achieve a final concentration of $2x10^6$ cells/100µl 1% agarose. The agarose plugs were allowed to set at 4°C for 10min and then lysed in L Buffer containing 1% SDS and 1mg/ml Proteinase K for 48h at 50°C.

2.2.1.3 Determination of DNA concentration

DNA concentrations were determined by Hoechst 33258 staining and spectrophotometric quantitation using a standard curve of human placental DNA.

2.2.2 Electrophoresis of DNA

2.2.2.1 Agarose gel electrophoresis

DNA samples and size markers, both in DNA loading buffer, were electrophoresed through 2% agarose gels containing 0.5μg/ml ethidium bromide. Gels were made by dissolving the appropriate amount of electrophoresis grade agarose in TBE. The gels were submerged in TBE and run at 3V/cm. Electrophoresed DNA was visualised using a UV transilluminator and a record of the gel was made using a digital imaging system.

2.2.2.2 Separation of DNA by field inversion gel electrophoresis

Approximately 10^6cells per lane were loaded onto a 1% electrophoresis grade agarose gel and the wells capped with 1% low gelling temperature agarose. Lambda Ladders and denatured λ DNA were run as size markers and untreated HL60 cells used as control. FIGE was carried out using a horizontal gel chamber, a 500V power supply and an IBI Minipulse Polarity Switching System. The gel was run at 100V in 50mM Tris-acetate, pH 7.7 and 0.5mM EDTA for 48h at 4°C with an initial reverse time of 0.1s; reverse increment, 0.02s and an initial forward time of 0.3s; forward increment, 0.06s.

2.2.3 Southern transfer of DNA

The DNA was then transferred onto nylon membrane by Southern blotting and the membrane hybridised with human and λ DNA randomly primed with (α^{32}P)dCTP.

2.2.4 Preparation of radiolabelled probes

Human double-stranded DNA was randomly sheared using a hypodermic needle and labelled with (α^{32}P)dCTP using the Prime-It kit (Stratagene, Cambridge, UK). Radiolabelled DNA was separated from unincorporated ^{32}P-labelled nucleotides using disposable sephadex G50 NICK columns (Pharmacia Biotech). The specific activity of radiolabelled probes was determined by taking a small aliquot (5µl) and measuring the number of disintegrations per minute using a liquid scintillation analyser (Packard, Pangbourne, Berkshire, UK). Probes were denatured before hybridisation by adding 0.1 volume of 3M NaOH and leaving for 5min at room temperature. After a 5min incubation on ice with 0.05 volume of 1M Tris.HCl (pH 7.5), a 0.1 volume of 3M HCl was added and left for a further 5min.

2.2.5 Hybridisation and washing of Southern blots

Pre-hybridisation of blots was carried out at 65°C in hybridisation buffer. Following hybridisation overnight at 65°C, filters were washed at 65°C using wash buffer until the background level of radioactivity was reduced sufficiently.

2.2.6 Extraction of protein

Cells were washed with PBS, then lysed at 4°C for 15min using high salt lysis buffer supplemented with protease inhibitors. The lysate was spun at 13,000rpm for 15min and the supernatant stored at -70°C.

2.2.7 Determination of protein concentration

Protein content was measured using the Bio-Rad kit method.

2.2.8 Denaturing gel electrophoresis of protein

Solubilised protein and size markers, were separated by SDS-PAGE on an 8% running gel after passing through a preliminary 4% stacking gel, both suspended in 1x tank buffer.

8% Running gel

8ml Running gel buffer

8.5ml 30% Acrylamide, 0.8% bis-acrylamide (Severn Biotech, Kidderminster, UK)

3.2ml 1% Polyacrylamide

12.3ml ddH_2O

120µl 10% APS

15µl TEMED

4% Spacer gel

3ml Spacer gel buffer

1.6ml 30% Acrylamide,0.8% bis-acrylamide

1.2ml 1% Polyacrylamide

4.8ml ddH_2O

180µl 10% APS

15µl TEMED

2.2.9 Western transfer of protein

Electroblotting was performed using a semi-dry electroblotter. Immobilon-P membrane (Millipore, Bedford, MA) was immersed in methanol and then transfer buffer. Six sheets of 3MM Whatman filter paper were sandwiched adjacent to the anode and cathode with the membrane and gel layered in between. Transfer took place over 1h at 200mA. After transfer, the gel was stained in Coomassie stain as described below. This allowed a visual assessment to be made of the uniformity of the transfer.

2.2.10 Coomassie staining of acrylamide gels

Gels were stained with Coomassie stain (0.2% Coomassie Brilliant Blue R250 in a 50:50:7 v/v ratio of methanol:H_2O:glacial acetic acid) for 4 hours and then de-stained using a 25:68:7 v/v ratio of methanol:H_2O:glacial acetic acid overnight.

2.2.11 Immunological detection of protein

Membranes were incubated with blocking solution at 4°C for 4h, probed overnight in the same buffer with primary antibody and washed with 0.1% Tween-20 in PBS. Blots were incubated in blocking solution with anti-mouse IgG HRP-linked rabbit antibody, then washed again in 0.1% Tween-20 in PBS, after which bound complexes were visualised by enhanced chemiluminescence (Boehringer Mannheim).

2.2.12 Autoradiography

Membranes were exposed to X-ray film (Fuji, supplied by H.A. West, Clydebank, Glasgow, UK) and were developed using a Kodak X-ray developer.

2.2.13 Tissue culture techniques

2.2.13.1 Aseptic technique

Aseptic manipulations were performed using sterilised glassware in a class II microbiological safety cabinet with vertical airflow. Cells were grown at 37°C as monolayers in supplemented RPMI 1640 (Rosswell Park Memorial Institute; see below for supplements) in the presence of 5% CO_2. They were stored by freezing in RPMI with 10% di-methyl sulphoxide (DMSO) in 1ml cryotubes at -70°C and were then maintained in liquid nitrogen. Mutant p53 and vector-alone transfected cells were regularly exposed to 400μg/ml G418 (Gibco BRL) to confirm the presence of the co-transfected G418 resistance plasmid.

RPMI

88 ml RPMI 1640 (10x)

800ml sterile distilled water

26.6ml 7.5% $Na(CO_3)_2$

10ml 100mM Na pyruvate

10ml 200mM L-glutamine

1ml 1M NaOH

100ml foetal calf serum

5ml penicillin / streptomycin (50mg)

Trypsin

20ml 10x PE

20ml trypsin (2.5% stock)

160ml sterile distilled water

PE

PBS

1mM EDTA

2.2.13.2 Treatment of cells with cytotoxic agents

Cells were treated on day 2 for clonogenic assays and on day 4 for TUNEL/PI staining.

2.2.13.2.1 Treatment with ionising radiation

All irradiations were performed using γ rays from an Alcyon II Teletherapy Unit ^{60}Co source at a dose rate of 2Gy per minute. Control cultures were kept in the ante-room of the cobalt source during irradiations. The growth medium was changed immediately before treatment and was not changed again during the course of the experiment.

2.2.13.2.2 Treatment with cisplatin

Cell cultures were treated with cisplatin from a stock dissolved in DMSO. The final concentration of DMSO in culture was 0.01%. Control cultures were treated

with 0.01% DMSO. Cells were treated for one hour after which, fresh medium was added. The medium was not changed again during the course of the experiment.

2.2.13.3 Clonogenic assay

Cells were seeded at $1x10^3$ per 90mm diameter dish on day 1. Colonies were grown for 10-14 days after treatment and then stained with 1 x Giemsa stain for 10 minutes and rinsed with tap water. Colonies of greater than 200 cells were counted.

2.2.13.4 Staining of cells

2.2.13.4.1 Staining with trypan blue

Cells were trypsinised, combined with suspension cells and resuspended in PBS containing 0.25% v/v trypan blue prior to counting using a hemocytometer.

2.2.13.4.2 Staining with acridine orange

Cells were trypsinised, combined with suspension cells, fixed in ethanol on ice and stored at 4°C. Cells were washed in PBS, dropped onto microscope slides, air dried and stained with 1μg/ml acridine orange in Vectashield anti-fade mountant H-1000 (Vector Labs, Peterborough, UK). Coverslips were sealed using nail varnish and the slides were stored in the dark at 4°C prior to analysis by confocal microscopy (see section 2.2.14.2).

2.2.14 Microscopy

2.2.14.1 Light microscopy

Light microscopy for routine culture of cells and for haemocytometer counts was performed using a Diavert Light Microscope .

2.2.14.2 Fluorescence microscopy

Fluorescence microscopy was used to visualise acridine orange-stained cells (see section 2.2.13.4.2) and cells stained by TUNEL (with FITC and PI). Small aliquots of TUNEL/PI-stained cells in suspension (20µl) were dropped onto microscope slides, air dried and mounted with Vectashield anti-fade mountant H-1000. Analysis of both staining procedures was performed on a laser scanning confocal microscope equipped with a krypton/argon ion laser. A 488/568nm line excitation filter and dual channel 522 and 585nm emission filters were used. Image analysis was performed using Bio-Rad software.

2.2.15 TUNEL assay

2.2.15.1 Harvesting of cells

Exponentially growing monolayer cells were trypsinised, combined with suspension cells and fixed in 1% formaldehyde for 15min. on ice. The cells were resuspended in PBS and 70% ethanol (pre-cooled to -20°C) and stored at 4°C prior to detection of non-random DNA strand breaks.

2.2.15.2 Detection of DNA strand breaks

Apoptotic cells were detected as described previously (Gorczyca et al., 1993). Cells were rehydrated in PBS and counted using a haemocytometer. Aliquots of 10^6 cells were incubated for 30min. at 37°C with cacodylate buffer containing 5units terminal DNA transferase (TdT), 0.5nmoles b-dUTP/10^6 cells. After washing in PBS cells were incubated for 30min. at room temperature in the dark with streptavidin-FITC. After a further wash in PBS and 0.1% Triton X-100 cells were resuspended in PBS and stained with 10μg/ml propidium iodide (PI).

2.2.15.3 Flow cytometry

Cellular fluorescence was detected using a FACScan flow cytometer (Becton Dickinson, Oxford, UK). After FITC staining, cells were washed with PBS and resuspended in 10μg/ml of PI in PBS and stored at 4°C until analysis. The red (PI) and green (FITC) emissions from each cell were separated and quantified using the standard optics of the FACScan. Analysis of data was carried out using the Cell Quest programme (Becton Dickinson).

CHAPTER 3

DEVELOPMENT OF METHODS FOR CONFIRMATION AND QUANTIFICATION OF APOPTOSIS

points and should ensure that all the apoptotic cells in the culture are collected on any one occasion.

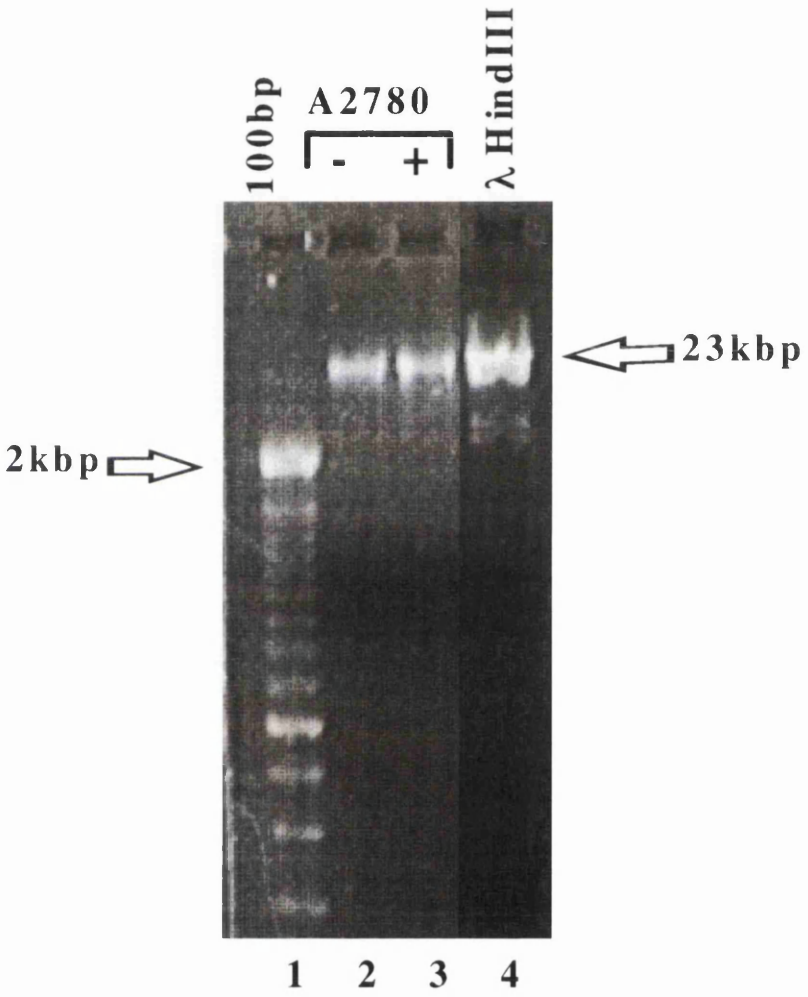
A2780 cells were irradiated with 20Gy ionising radiation. Unirradiated cells were used as controls. The suspension cells were harvested at 24h, 48h and 72h after irradiation and DNA was prepared from the total suspension cells collected at these three time points as described in section 2.2.1.1. The DNA samples from irradiated and unirradiated suspension cells were electrophoresed according to the protocol outlined in section 2.2.2.1.

No evidence of internucleosomal, non-random DNA fragmentation was observed in the DNA preparations, whether isolated from the suspension cells of irradiated or unirradiated cultures (Figure 3.1). The smallest size of DNA fragment seen under these conditions was approximately 23kbp in length, whereas when internucleosomal DNA laddering is observed in apoptotic mammalian cells the smallest size of DNA fragment that can be detected is approximately 150-200bp (Walker et al., 1993).

3.2.2 Southern blotting of electrophoresed DNA samples

No DNA laddering was observed by ethidium bromide staining in the above samples and therefore it would appear that no apoptosis was occurring. The number of apoptotic cells in a culture at any one time may be very low since the execution of apoptosis is rapid and the time over which one can detect any one particular apoptotic cell may be as little as 30 minutes (Evan et al., 1992) thus resulting in a low yield of apoptotic cells. In an attempt to sensitise the electrophoretic detection of DNA from apoptotic cells, the same samples as used in section 3.2.1 (DNA extracted from suspension cells from unirradiated and 20Gy irradiated A2780 cultures) were electrophoresed on a large agarose gel. The DNA was then Southern blotted onto

Figure 3.1 Agarose gel of DNA from suspension cells of A2780 cultures. Lane 2, unirradiated (-) A2780 suspension cells; lane 3, 20Gy irradiated (+) A2780 suspension cells. Size markers are in lane 1 (100 base pair DNA ladder) and lane 4 (HindIII digestion of λ DNA).



Hybond-N nylon membrane and hybridised with randomly-sheared, human DNA which was ^{32}P-labelled by random priming (see sections 2.2.3 to 2.2.5). This allows visualisation of the total human DNA that is present on the membrane due to hybridisation of the probe to repetitive DNA sequences. Again, no evidence of DNA laddering was observed (data not shown).

3.3 Field inversion gel electrophoresis and Southern blotting for detection of 50kbp and 300-1000kbp DNA fragments

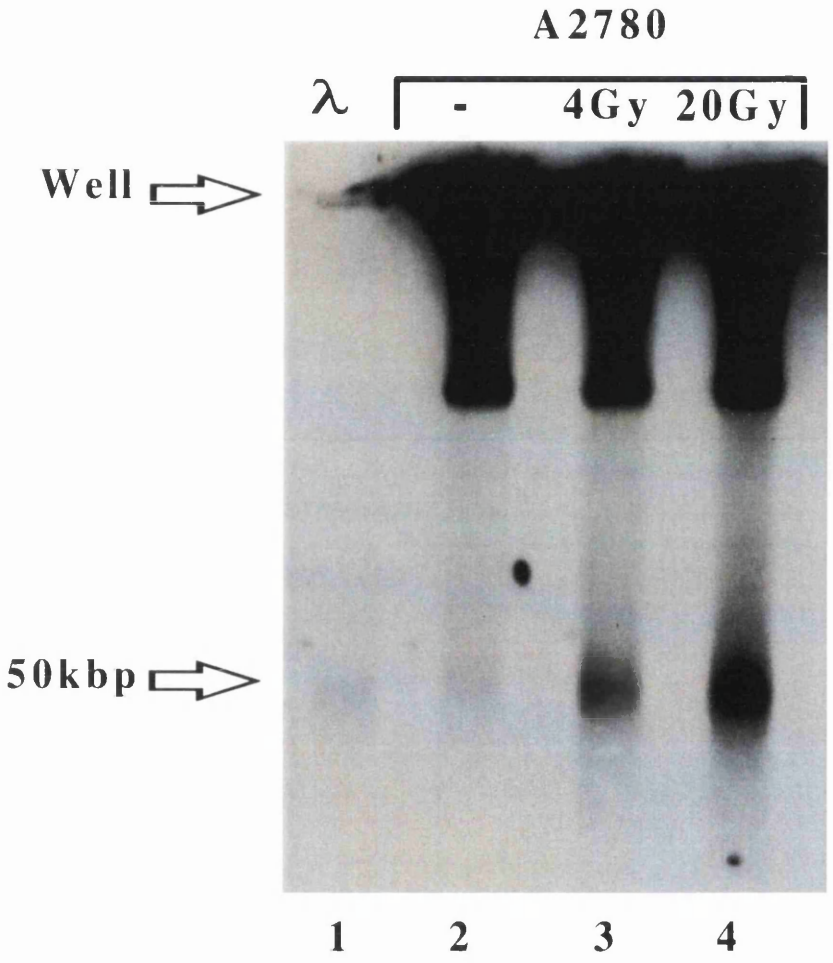
It has been found that only some cell lines undergo non-random internucleosomal DNA laddering during apoptosis. Those that do not may still become apoptotic, however in these cases the DNA is degraded in a non-random fashion to produce DNA fragments of two sizes: one of 50kbp and the other of approximately 300-1000kbp (Oberhammer et al., 1993). Normal agarose gel electrophoresis cannot detect fragments of this size and so I used field inversion gel electrophoresis (see section 2.2.2.2) in conjunction with Southern blotting and detection of DNA with radiolabelled probe as described above. Initially I used DNA extracts from cell cultures however these were susceptible to random degradation and shearing during handling resulting in a smear of DNA on the autoradiograph. To minimise random shearing I adopted the method of embedding cells in agarose plugs before lysing to liberate the DNA. In an attempt to produce more quantitative results, total cell cultures (suspension cells and trypsinised, monolayer cells) were resuspended in molten agarose at a concentration of 10^6 cells/50µl. An approximately equal volume of 50µl was then loaded in each well (10^6 cells/well) thereby allowing the detection of apoptotic DNA fragments relative to the total amount of DNA obtained from the culture.

Field inversion gel electrophoresis enabled discrimination between genomic DNA and 50kbp DNA fragments in irradiated A2780 cells. Figure 3.2 shows an irradiation dose-dependent induction of DNA fragmentation in A2780 cells as measured by the appearance of 50kbp fragments. It must be stressed that due to the small volumes used to resuspend the cells (relative to the size of the cell pellet) this measure of programmed cell death is of limited quantitative value and therefore it was necessary to develop a protocol to allow detection and counting of apoptotic cells.

3.4 Acridine Orange staining

Many investigators count apoptotic cells by staining with the nucleic acid specific stain, acridine orange. Apoptotic cells stained in this way appear small and round with brightly stained nuclei due to highly condensed chromatin. In addition, the chromatin may be arranged in the characteristic shape of a crescent with a dark shadow along it's outer edge. I stained irradiated A2780 cells with acridine orange (see section 2.2.13.4.2) and attempted to score for apoptosis on the basis of differential morphology. A2780 cells however, are normally small and round and do not flatten out to any great extent on the monolayer. They also did not display a very obvious changed nuclear morphology and it was difficult in some cases to distinguish between mitotic and apoptotic cells. For these reasons I decided that acridine orange staining would not provide a sufficiently objective method for quantitating apoptosis in these cells lines.

Figure 3.2 FIGE of unirradiated (-), 4Gy and 20Gy irradiated A2780 cells (total cell population, 72h after treatment). Lane 1 contains full length, linearised Lambda DNA.



3.5 Measurement of live cells by trypan blue exclusion

The plasma membranes of apoptotic cells remain intact until the very advanced stages of the process and cells are still alive despite being committed to a cell death pathway culminating in apoptosis. Trypan blue can only enter cells whose membranes are permeable. If the apoptotic cells detach from the monolayer it should be possible to determine the number of apoptotic cells in the supernatant of a monolayer culture by counting those cells that exclude trypan blue. This method should therefore differentiate between cells found in the suspension whose membranes are intact and thereby exclude trypan blue (apoptotic cells) and those whose membranes are permeable and which thereby stain blue (very late-stage apoptotic cells, apoptotic bodies and cell debris).

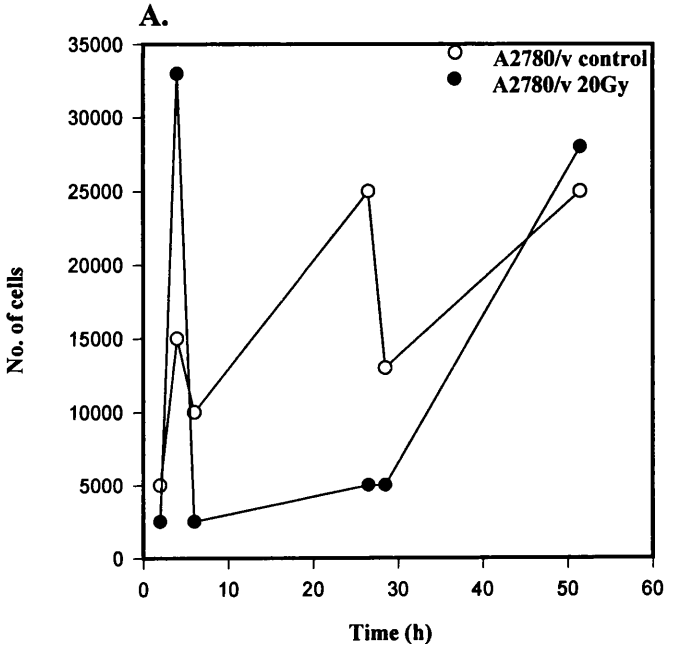
Figure 3.3 shows the results of cell counts based on trypan blue exclusion from suspension cells of irradiated A2780/v and A2780/mp53 cultures (see section 2.2.13.4.1 for staining protocol). For the purpose of determining the feasibility of this method, only suspension cells were considered and the adherent population (and therefore the total population) was not counted. Therefore conclusions can only be drawn on the difference between untreated and treated populations of the same cell line. No comparisons can be made between the two cell lines based on the percentage apoptotic cells in the total populations.

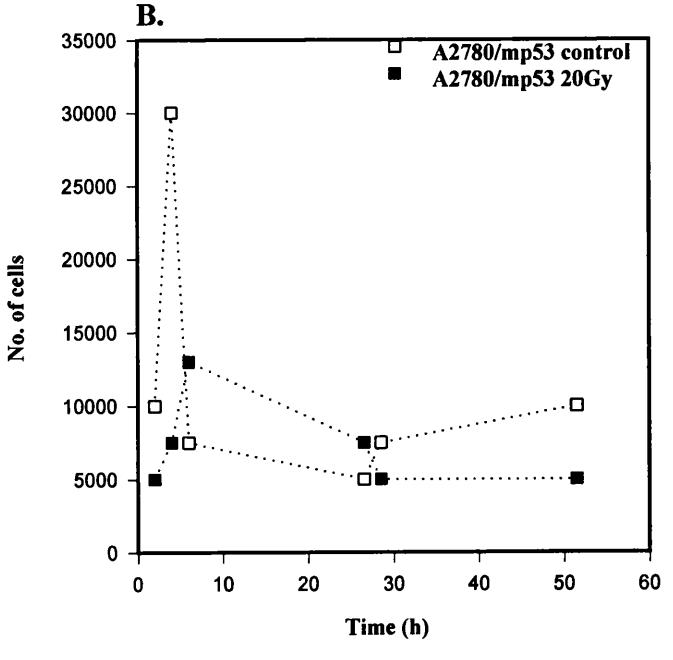
There was approximately a 2 fold higher level of trypan blue excluding A2780/v cells in the irradiated culture at 4h after treatment compared with unirradiated cells (Figure 3.3A). At all other time points however, there was either little difference between irradiated and unirradiated A2780/v cells (2h and 52h) or more trypan blue excluding cells in the unirradiated control (6h, 36h and 38h).

Figure 3.3 Number of live cells in supernatant of unirradiated and 20Gy irradiated cultures as measured by trypan blue exclusion.

A. A2780/v

B. A2780/mp53





There were more trypan blue excluding cells in the unirradiated A2780/mp53 culture at all time points except at 6h and 26h at which times there were only slightly more trypan blue excluding cells in the treated culture (Figure 3.3B).

As a result of later experiments it became apparent that significant levels of ionising radiation-induced apoptosis could not be detected in these cell lines at times earlier than 48h (see Chapter 4, section 4.3 and 4.4) and so this could account for the lack of increased trypan blue excluding cells in the suspensions of treated compared to untreated cultures. This could not however, account for the higher number of trypan blue excluding cells in control cultures at many of the time points. In addition, due to the lack of total cell counts and thereby the lack of percentage values it was impossible to judge whether this higher level of trypan blue excluding cells in control cultures was significant or not.

It would appear that the A2780 cell line and its derivatives are loosely adhering, anchorage-dependent cell lines. This may account for the spurious results obtained using this method in that some of the cells collected from the suspension of the culture will be non-apoptotic, mitotic 'knock-off' cells. These results clearly demonstrate that levels of apoptosis cannot be measured simply by counting those trypan blue excluding cells that detach from the monolayer. No conclusions could be drawn concerning the ability of ionising radiation to induce apoptosis in these cell lines or concerning the differences in apoptotic response between the two lines.

3.6 Fluorescent end-labelling of non-random DNA fragmentation for flow cytometric analysis of apoptosis

3.6.1 Theory

Apoptotic DNA fragments can be detected using terminal DNA transferase (TdT) to end label DNA strand breaks with a fluorescent tag (see section 2.2.15). The amount of DNA fragmentation in individual cells is proportional to the green fluorescent signal from the FITC-labelled DNA strands. The apoptotic cells in a population are those in which an increased level of green fluorescence is detected. Figure 3.4 shows a typical flow cytometry profile of cells labelled in this way. Propidium iodide staining (x-axis) detects total DNA and facilitates the visualisation of the G1, S and G2 populations. Figure 3.4B shows A2780 cells which were treated for 1h with 40μM cisplatin (also see section 5.4). The apoptotic cells, with high green fluorescence (y-axis) can be distinguished from the untreated cells with lower green fluorescence. This method is also known as TUNEL staining (TdT-mediated b-dUTP nick end labelling).

3.6.2 Controls

A number of controls were performed in order to insure that the staining was giving a true measure of apoptosis in the treated cells compared with untreated cells. Figure 3.5 shows the flow cytometry dot plots obtained from these controls. A2780 cells were found to have no detectable intrinsic fluorescence (A). Staining with TdT alone did not confer any fluorescent property on the cells (B) and there was no non-specific binding of FITC-streptavidin (C). Untreated cells, when stained with all of the components necessary to end-label strand breaks but omitting the propidium iodide (PI) did not display any significant FITC fluorescence, as would be expected of healthy cells

Figure 3.4 An example of a contour plot obtained by TUNEL/PI staining followed by FACs analysis. Fluorescence from propidium iodide is detected in the FL2 channel (x axis) and fluorescence from FITC staining is detected in the FL1 channel (y axis).

A. A2780 cells 72h after no treatment

B. A2780 cells 72h after 40μM cisplatin

C. A2780/cp70 cells 72h after no treatment

D. A2780/cp70 cells 72h after 40μM cisplatin.

These results are discussed in Chapter 5.

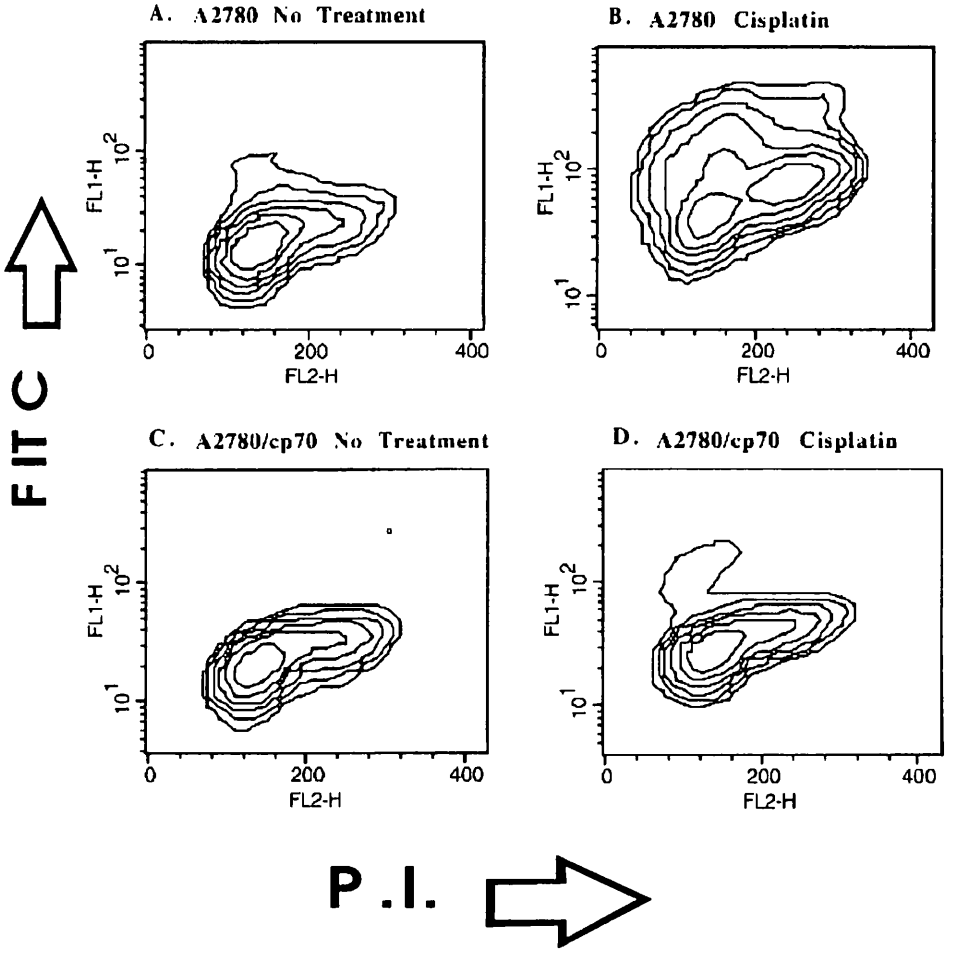
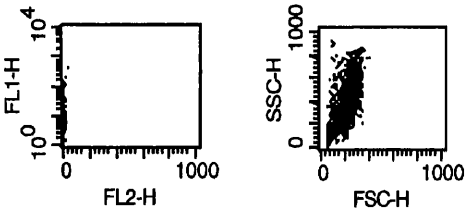


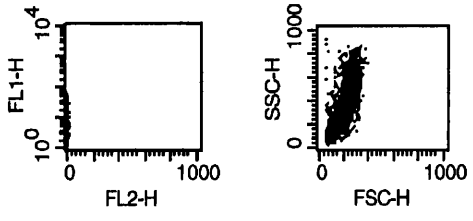
Figure 3.5 Controls for specificity of fluorescence in TUNEL/PI-stained cells

The plots on the left of each panel display PI fluorescence (FL2-H, x-axis) and FITC fluorescence (FL-1, y-axis). The plots on the right display forward light scatter (FCS-H, x-axis) which is a measure of cell size and side scatter of laser light (SSC-H, y-axis) which is a measure of cell granularity. This latter plot was used to detect the cell population which was then gated using the CellQuest package. The gated population was used for all subsequent analysis. This means that the cells which were analysed were of normal size and abnormally large cells were thereby excluded from the analysis. Cells which are dying by apoptosis are either of normal size or smaller. Cells which swell may be dying by other modes of cell death such as necrosis. This approach was employed as a way of excluding cells which may have been dying by modes of cell death other than apoptosis.

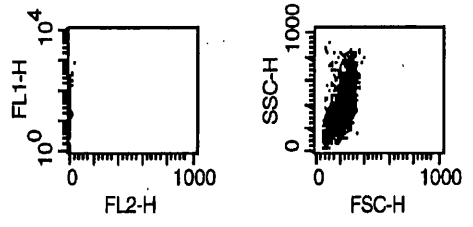
A. Healthy cells: no TdT, no b-dUTP, no FITC-streptavidin, no PI



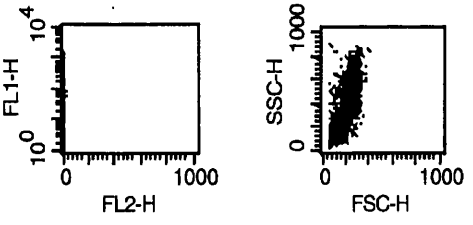
B. Healthy cells: TDT only, no b-dUTP, no FITC-streptavidin, no PI



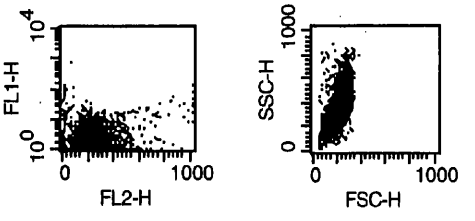
C. Healthy cells: FITC-streptavidin only, no TdT, no b-dUTP, no PI



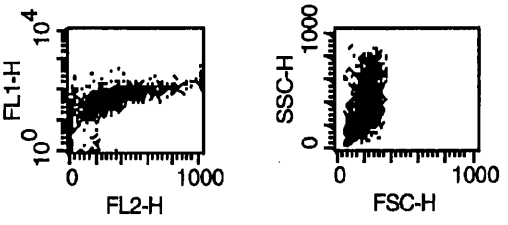
D. Healthy cells: TdT, b-dUTP & FITC-streptavidin, no PI



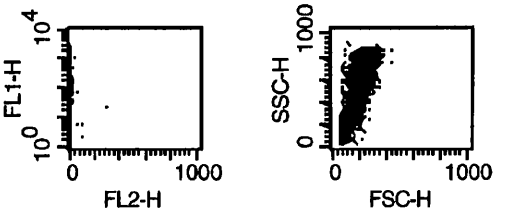
E. Healthy cells: PI only, no TdT, no b-dUTP, no FITC-streptavidin



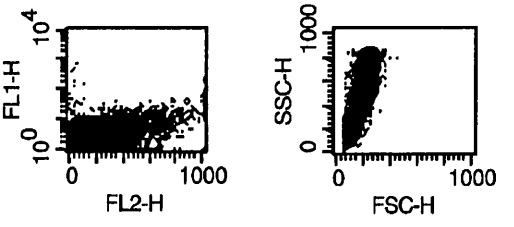
F. Healthy cells: TDT, b-dUTP, FITC-streptavidin & PI (all components)



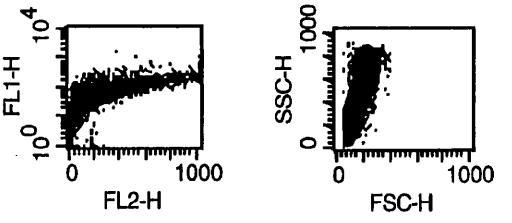
G. Cells with strand breaks: TdT, b-dUTP & FITC-streptavidin, no PI



H. Cells with strand breaks: PI only, no TdT, no b-dUTP, no FITC-streptavidin



I. Cells with strand breaks: TdT, b-dUTP, FITC-streptavidin & PI (all components)



(D). Corresponding cisplatin-treated cells (72 hours after treatment) did exhibit an FITC signal as would be expected if apoptotic cells were present (G).

Fluorochromes emit light over a range of wavelengths. When cells are stained with two or more fluorochromes the signal from one fluorochrome may overlap with that of another. The detector used for the first fluorochrome may detect signal from the second. It is important to insure that the FITC detector does not pick up signals from PI (and vice versa) leading to the detection of artificially high levels of either FITC or PI fluorescence. In cells stained with PI alone, PI fluorescence did not contribute significantly to the FITC signal (E and H). Likewise, in treated cells stained with all of the components except PI, FITC fluorescence was detected but it did not overlap into the range of signals detected by the PI detector (G). Panels F and I show the dot plots for untreated and treated cells respectively, stained with all of the components.

To insure that the staining was measuring fluorescence due to apoptosis and not due to initial DNA strand breaks caused directly by ionising radiation, trypsinised A2780 and A2780/cp70 cells were irradiated with 4Gy, put immediately on ice to prevent apoptosis from occurring and were fixed and stained. No increase in FITC fluorescence was observed in irradiated A2780 or irradiated A2780/cp70 cells compared to their respective unirradiated controls. Thus, a dose of 4Gy ionising radiation did not cause a sufficient number of strand breaks in either cell line to be detected by TUNEL/PI staining.

For analysis the profiles were displayed in contour plot form (as in Figure 3.4) in order to distinguish more easily between apoptotic and non-apoptotic cell populations. This method proved to be the most reliable and least subjective technique for quantitating apoptosis in these cells. Results from ionising radiation- and cisplatin-

induced apoptosis studies as measured by this method are presented in chapters 4 and 5 respectively.

3.6.3 Cellular morphology and detection of fluorescent end-labelling by microscopy

A2780 and A2780/cp70 cells which were stained using TUNEL/PI were dried onto microscope slides and visualised by confocal microscopy (see Section 2.2.14.2). Typical fields of view are shown in Figure 3.6. Images were saved and a software program (PC Ratio, Beatson Laboratories, Glasgow) was used to measure the total pixel intensity of the PI and FITC signals from individual cells. This data was plotted on a dot plot (Figure 3.7). In Figure 3.6B it can be seen that those regions which visibly stained green (due to FITC) seemed to be apoptotic bodies and cells with apparent apoptotic morphology. Furthermore it can be seen from Figure 3.7 that the treated A2780 cell population contained a higher proportion of cells with increased FITC fluorescence than did the untreated A2780 cell population or the A2780/cp70 cell populations (treated or untreated). To score the cells by eye would be subjective and time consuming. In addition the number of cells that could reasonably be counted would be only 200-300 cells per slide compared with 15,000 cells that can easily be analysed by flow cytometry. Analysis of the TUNEL/PI-stained cells by confocal microscopy confirmed that the cells which stained with FITC were analogous to the apoptotic cells and that the staining method can be used to distinguish between cells which do and do not undergo apoptosis in response to DNA damage.

Figure 3.6 Photographs of cells obtained by confocal imaging.

A. A2780 cells 72h after no treatment

B. A2780 cells 72h after 20μM cisplatin

C. A2780/cp70 cells 72h after no treatment

D. A2780/cp70 cells 72h after 40μM cisplatin

The arrows mark cells in the late stages of apoptosis, showing green fluorescence due to end-labelling of DNA strand breaks by TUNEL (1) and apoptotic bodies (2).

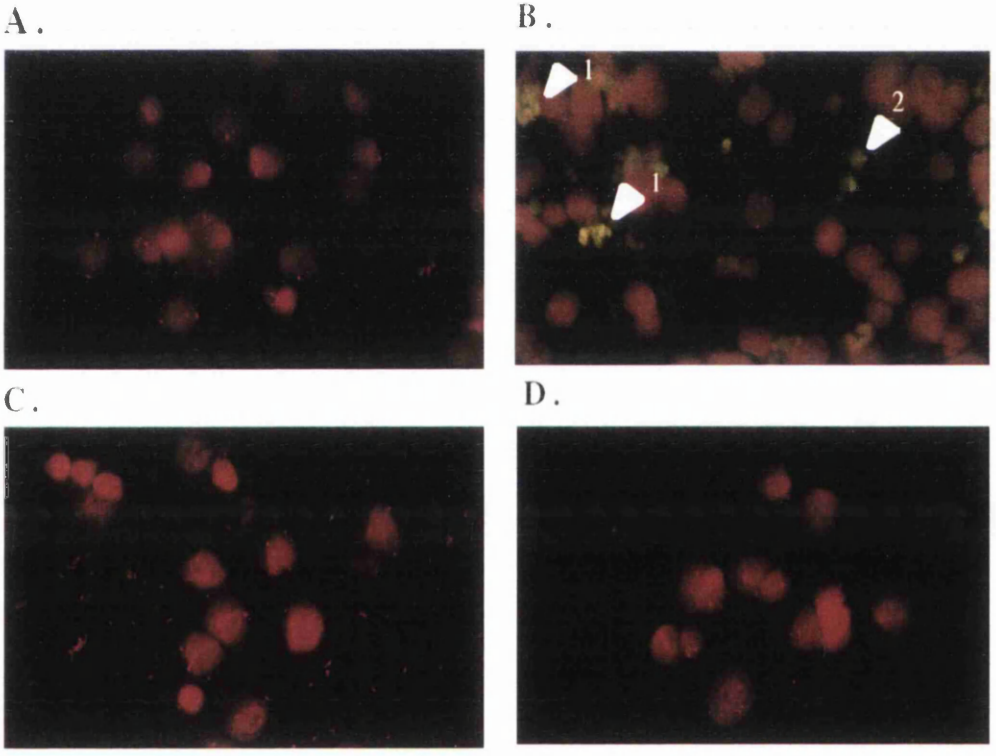
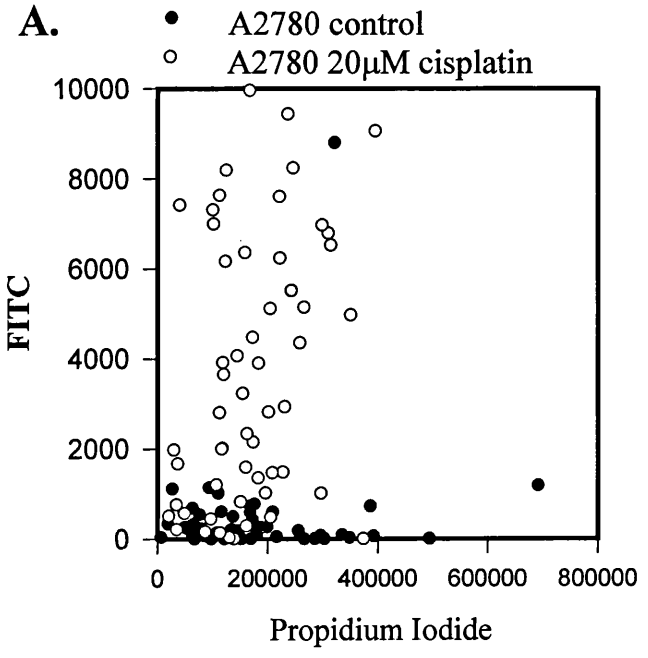
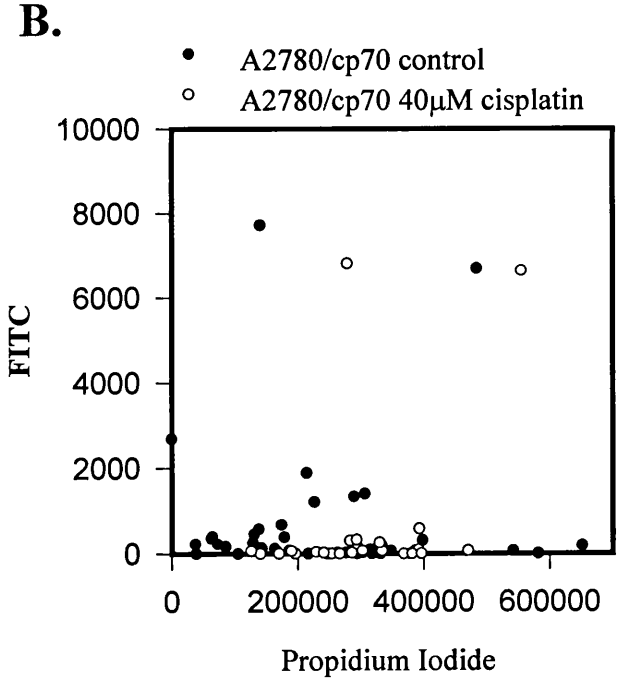


Figure 3.7 Dot plots displaying the data obtained from confocal image analysis of cells shown in Figure 3.6 (72h after cisplatin treatment). Units of fluorescence represent total pixel intensity per cell.





3.7 Conclusions

In summary, the A2780 cell line and it's derivatives did not undergo detectable internucleosomal DNA laddering in response to ionising radiation (or cisplatin, see Chapter 5). Their DNA did degrade to fragments of approximately 50 and 300-1000kbp in length which is characteristic of apoptotic cells. To separate these fragments it was necessary to subject the DNA preparations to field inversion gel electrophoresis. There was clearly a low level of apoptosis occurring and so ethidium bromide staining was insufficient to detect the DNA fragments. Thus Southern blotting followed by hybridisation of randomly sheared, radiolabelled human DNA was employed as a more sensitive method of detection.

The morphology of normal A2780 cells was such that simple staining with acridine orange in order to identify and count cells with apoptotic morphology was difficult and highly subjective. The loosely adherent nature of the cells also made counting live suspension cells by trypan blue exclusion an invalid method for quantitating apoptosis. Thus the TUNEL/PI staining method in conjunction with flow cytometry was used as it provided a rapid, less subjective measure of apoptosis in large populations of cells.

Field inversion gel electrophoresis and TUNEL/PI staining were subsequently used to identify and quantitate ionising radiation- and cisplatin-induced apoptosis in the A2780 cell line and it's derivatives and to attempt to determine the extent of the dependence of the apoptotic response on functional p53. The results of these studies are reported in the following two chapters.

CHAPTER 4

IONISING RADIATION-INDUCED APOPTOSIS IN HUMAN OVARIAN CELL LINES

4.1 Introduction and Aims

A2780/cp70 cells are resistant to cisplatin, ionising radiation and multiple chemotherapeutic agents compared to the parental A2780 cells as measured by clonogenic survival assay (Vasey et al., 1996; Behrens et al., 1987). In addition ionising radiation and cisplatin cause an inhibition of DNA synthesis (G1 arrest) and a G2 cell cycle arrest in A2780 cells, whereas A2780/cp70 cells only arrest in the G2 phase (Brown et al., 1993). Transfection of the A2780 cell line with a dominant negative mutant of *TP53* (codon 143 val-ala) causes an abrogation of the ionising radiation-induced G1 arrest (Figure 4.1). In addition these mutant p53 transfectants are more resistant to ionising radiation and cisplatin (Vasey et al., 1996; McIlwrath et al., 1994). Vector-alone transfectants are comparable to the parental cell line in terms of their sensitivity to these two chemotherapeutic agents and their capability to arrest in the G1 and G2 phases of the cells cycle. Thus the ionising radiation-induced inhibition of DNA synthesis observed in A2780 cells is a p53-dependent cell cycle arrest and the sensitivity of A2780 cells to ionising radiation is at least in part due to the presence of a functional p53 protein. Both A2780 and A2780/cp70 contain wild type *TP53* gene sequence (Brown et al., 1993) however it would appear that the p53 protein which is present in A2780/cp70 cells is dysfunctional.

I therefore compared levels of ionising radiation-induced apoptosis between the cell lines to ask the question: does the ability to engage apoptosis play a role in DNA damage-induced cytotoxicity by ionising radiation and to what extent does the apoptotic response depend on the presence of functional p53?

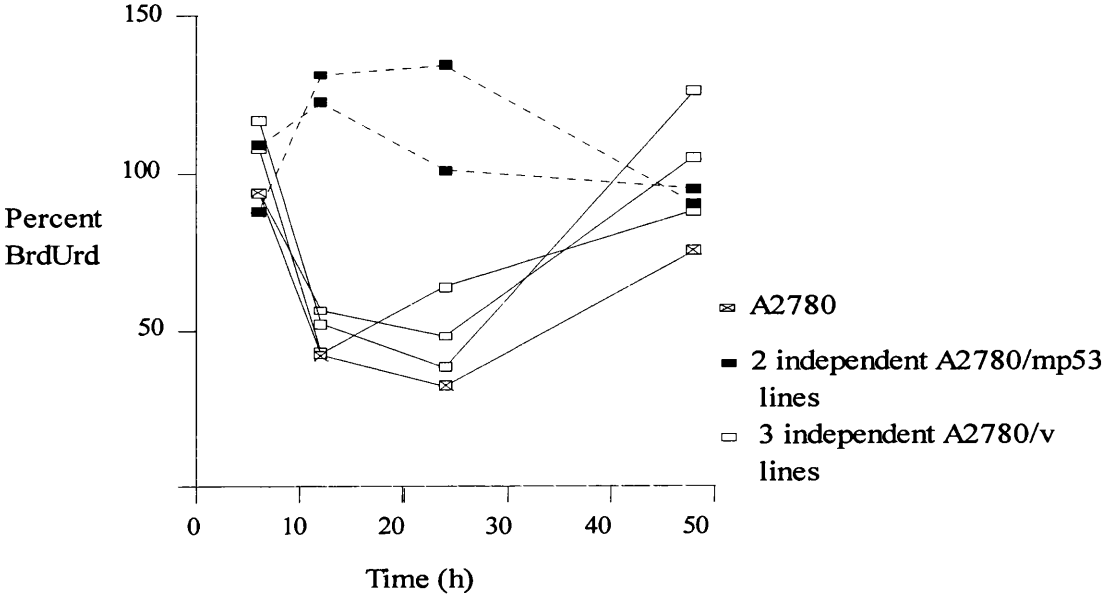
Figure 4.1 Loss of p53 function in mutant TP53 transfectants.

A. Ionising radiation-induced inhibition of DNA synthesis as measured by BrdU incorporation into vector alone transfectants (A2780/v) and dominant negative mutant p53 transfectants (A2780/mp53)

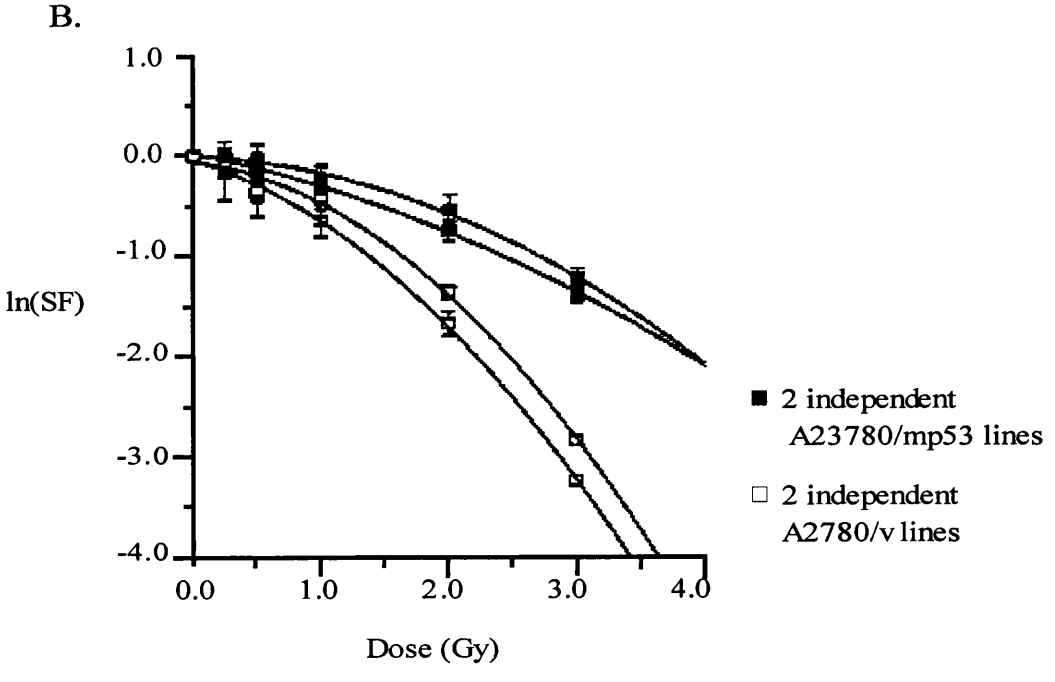
B. Ionising radiation survival curves for the transfectants as measured by clonogenic survival assay

(Reproduced from McIlwrath et al., 1994)

A.



B.



4.2 Clonogenic survival of vector alone and mutant p53 transfectants of A2780

The clonogenic survival data shown in Figure 4.1B was obtained by irradiating cells seeded at a low density of 10^3 cells per plate (see section 2.2.13.3). The survival curve for A2780/mp53 cells is shifted to the right indicating increased resistance of these cells to ionising radiation as measured by clonogenic assay. The surviving fractions after 2Gy irradiation (SF2Gy) for the two mutant p53-transfected cell lines in Figure 4.1B were 0.58 and 0.47 (mean=0.53). For the two vector-transfected cell lines the SF2Gy values were 0.26 and 0.18 (mean=0.22). This translates as a 2.4-fold resistance to ionising radiation in the mutant p53 cells compared to the vector control cells. To measure apoptosis by either FIGE or flow cytometry, the cells must be seeded and treated at high density (approximately 10^6 cells per plate or greater at the time of treatment). I therefore performed a clonogenic survival assay after irradiation of cells growing at high density and compared the SF2Gy values and fold resistance with those obtained from a low cell density clonogenic assay.

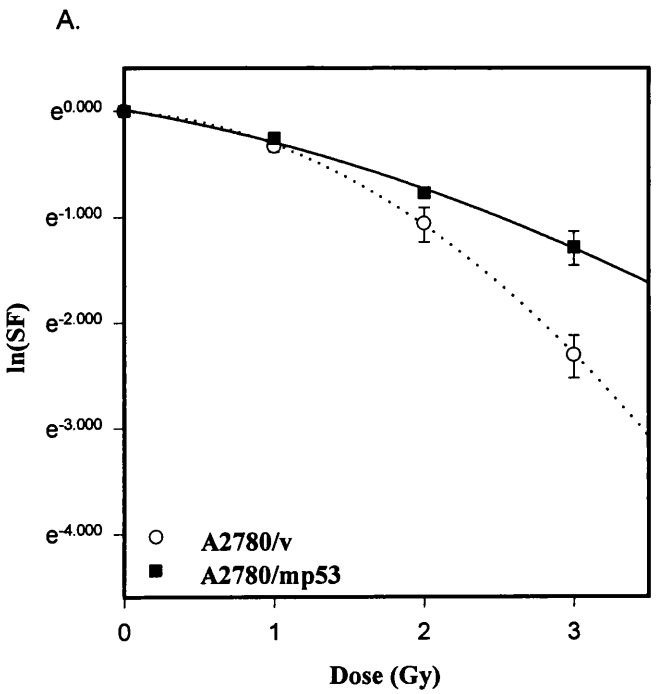
Exponentially growing cells were irradiated at a density of at least 10^6 cells per plate. The cells were then re-seeded at 10^3 per plate and colonies were allowed to form. A low density clonogenic assay was done in parallel. Figure 4.2 shows the data from these clonogenic assays. Plating efficiencies of control cells were 38% for A2780/v and 23% for A2780/mp53 in the low density assay and 38% and 28% respectively in the high density clonogenic assay. The difference in the plating efficiency of A2780/mp53 cells between the two assays most likely reflects a variation in cell counts. It is clear though, that in both cases the plating efficiency of A2780/mp53 cells is reduced compared with A2780/v cells. Following irradiation at low density (Figure 4.2A) the SF2Gy value for A2780/v cells was 0.34 while the SF2Gy for the resistant A2780/mp53 cells was 0.5.

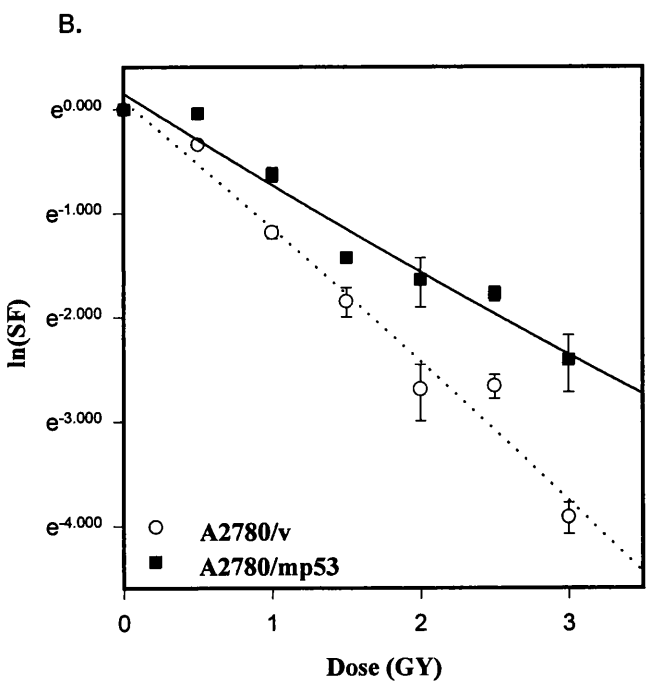
Figure 4.2 Clonogenic survival curves of irradiated cells.

A. cells irradiated at low density

B. cells irradiated at high density and replated

Error bars represent the standard error of the mean; n=3.



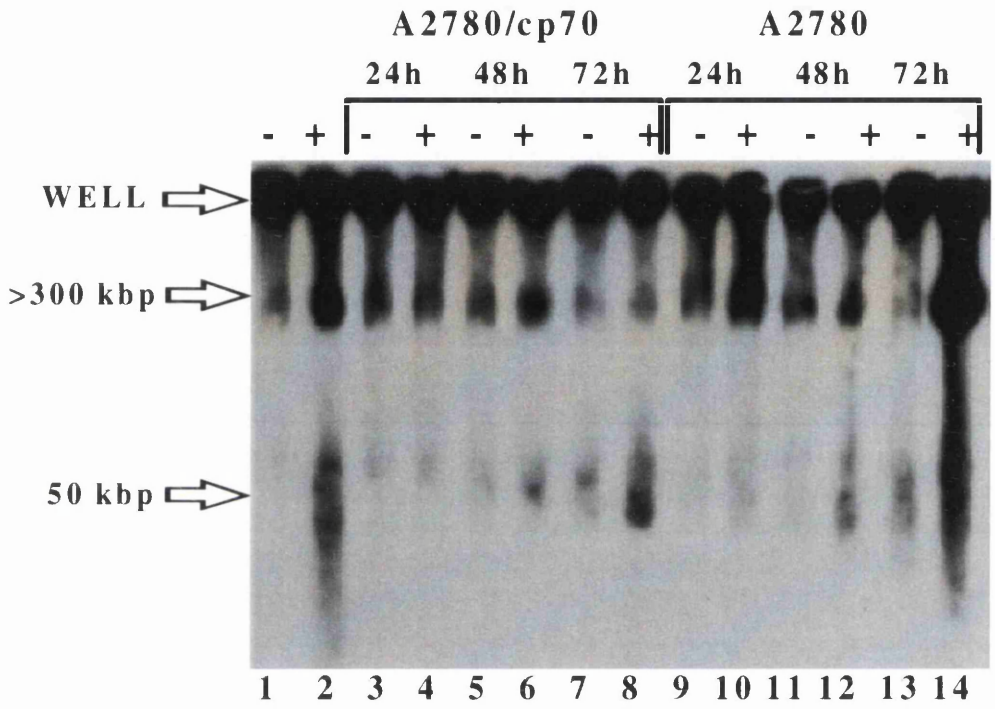


The surviving fraction of cells irradiated at high density (Figure 4.2B) was lower for both A2780/v (SF2Gy=0.1) and A2780/mp53 (SF2Gy=0.21) compared to cells irradiated at low density. Cells which have been recently irradiated are perhaps more susceptible to the additional stress of trypsinisation than unirradiated cells and while the control cells can be trypsinised and replated without significantly affecting subsequent plating efficiency, the plating efficiency of irradiated cells may be reduced thereby reducing the fraction that form colonies relative to control. The fold resistance of A2780/mp53 compared with A2780/v cells however, did not differ greatly between the two assays (1.5 fold resistant at low density and 2 fold resistant at high density). It was therefore valid to investigate the role of apoptosis in resistance by irradiating cells at a high density.

4.3 Production of large apoptotic DNA fragments in A2780 and A2780/cp70 in response to ionising radiation

A2780 and A2780/cp70 cells were irradiated with 4Gy ionising radiation, embedded in agarose and lysed (see section 3.3). This dose is equally damaging to both cell types, introducing an equal number of strand breaks in both A2780 and A2780/cp70 cells (Brown et al., 1993). Figure 4.3 shows the autoradiograph obtained. At 24h and 72h after irradiation there was an increase in the amount of the larger (300-1000kbp) DNA fragment in A2780 cells compared with unirradiated A2780 cells (lanes 9&10 and 13&14) and at 48h and 72h there was also an increase in fragments of 50kbp in size in the irradiated cells (lanes 11&12 and 13&14). A2780/cp70 cells showed an increase in the appearance of 300-1000kbp DNA fragments 48h after irradiation compared with controls (lanes 5&6) and a slight increase in the appearance of 50kbp fragments at 72h (lanes 7&8). While no firm conclusions could be drawn concerning the quantitation of

Figure 4.3 Autoradiograph showing results of FIGE of DNA from unirradiated (-) and 4Gy irradiated (+) A2780 (lanes 9-14) and A2780/cp70 cells (lanes 2-8). The negative control (lane 1) is unirradiated HL60 cells. The positive control (lane 2) is the 4Gy irradiated A2780 DNA sample shown in Figure 3.2.



apoptosis based on these results due to slight variations in DNA loading, it would appear that the A2780 cells underwent apoptosis more readily than did A2780/cp70 cells in response to equally damaging doses of ionising radiation.

4.4 Percentage ionising radiation-induced apoptosis in A2780 and A2780/cp70

A2780 cells were irradiated with either 2Gy or 4Gy ionising radiation and A2780/cp70 cells were irradiated with 4Gy. This approach was used to compare the responses of the two cell lines to equally damaging doses of irradiation (4Gy) and to compare the effect of doses which produced approximately equal surviving fractions of 0.2 as measured by clonogenic assay (2Gy for A2780 and 4Gy for A2780/cp70, Brown et al., 1993). In order to disrupt the cells as little as possible throughout the time course, no medium changes were performed. Cells were detected by TUNEL and propidium iodide staining (see Section 2.2.15). Figure 4.4 shows photographs of control and irradiated cells acquired using confocal microscopy. Typical apoptotic morphology (condensed chromatin and apoptotic bodies) and increased FITC fluorescence (DNA fragmentation) can be seen in the irradiated A2780 cells (Panels B and C).

The stained cells were detected by flow cytometry and the data for four repeat experiments (three repeats for 2Gy irradiated A2780) is shown in Figure 4.5. There was a low background level of apoptosis in untreated cells. The high level of apoptosis seen in unirradiated A2780 cells at 72h was not consistently observed but was due to a high percentage of apoptotic cells in the control of 1 out of 4 repeat experiments.

After irradiation of both cell lines with 4Gy (inducing equal initial damage in each line) apoptosis was 5 fold higher in A2780 compared with A2780/cp70 at 24h and 48h. Apoptosis was 6 fold higher in A2780 compared with A2780/cp70 at 72h and 7 fold

Figure 4.4 Confocal microscopy images of A2780 and A2780/cp70 cells stained by TUNEL/PI for flow cytometric detection of apoptosis.

A. A2780 cells 72h after no treatment

B. A2780 cells 72h after 2Gy ionising radiation

C. A2780 cells 72h after 4Gy ionising radiation

D. A2780/cp70 cells 72h after no treatment

E. A2780/cp70 cells 72h after 4Gy ionising radiation.

Apoptotic cells are those with green staining due to end labelling of DNA strand breaks by the TUNEL method. Apoptotic morphology is visable in the ionising radiation-treated A2780 cells. 1, pyknotic nuclei with condensed chromatin around the nuclear margin; 2, cells with fragmented chromatin within their nuclei; 3, apoptotic bodies.

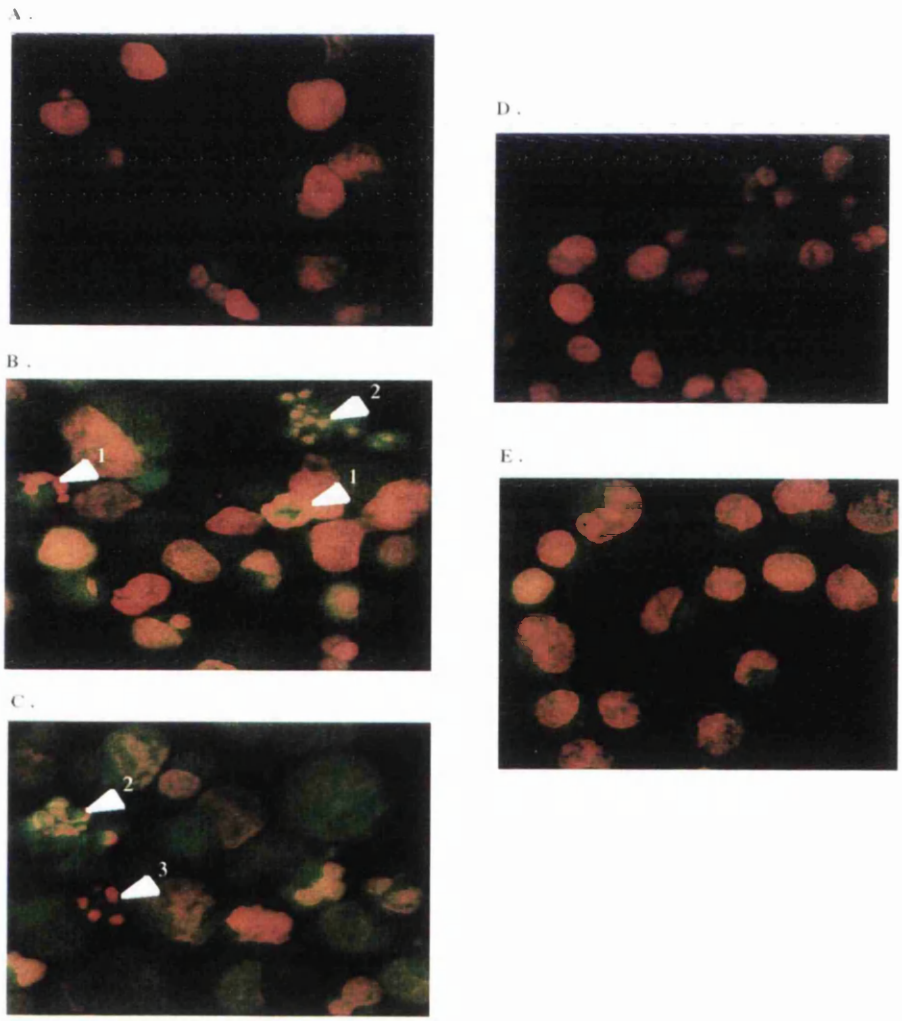
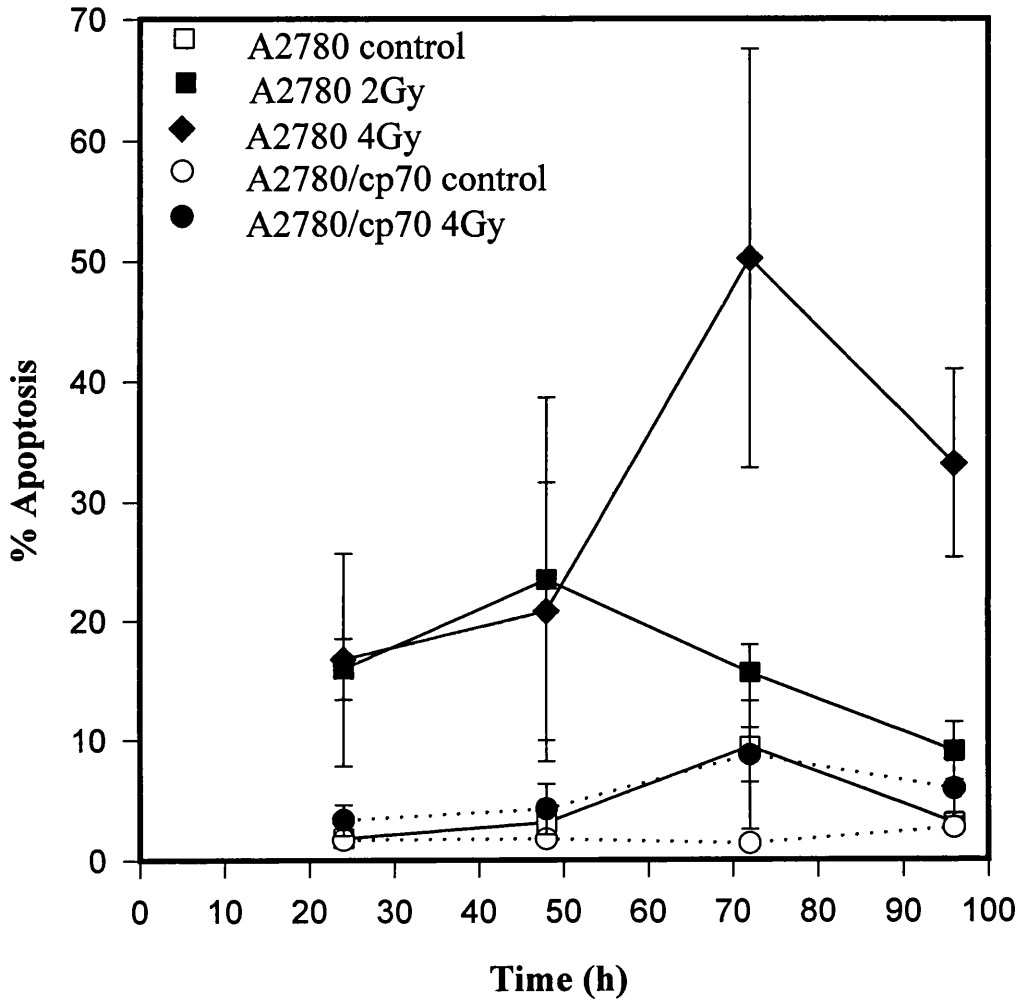


Figure 4.5 Percentage ionising radiation-induced apoptosis in A2780 (solid lines) and A2780/cp70 cells(dotted lines) as measured by TUNEL/PI staining and FACs analysis. Error bars represent the standard error of the mean of at least three duplicate experiments.



higher at 96h after 4Gy. Approximately the same fold increases in apoptosis (5 fold) were observed in A2780 cells at 24h and 48h after 2Gy ionising radiation compared with 4Gy irradiated A2780/cp70 cells (equitoxic doses). At both 72h and 96h after equitoxic doses of irradiation apoptosis was 2 fold higher in A2780 compared to A2780/cp70. It would therefore appear that A2780/cp70 cells have a reduced ability to engage apoptosis in response to ionising radiation compared with A2780 cells, even after a dose of 4Gy which produces colony forming efficiency equal to that for 2Gy irradiated A2780 cells.

The differences in apoptosis induction between the two cell lines were tested for statistical significance using the Students *t*-test. The statistical tests were performed by pooling the data from the 72h and 96h time points which, in the majority of experiments, were the time points at which the maximal levels of apoptosis were observed. The P values calculated using this test are presented in Table 4.1. The same criteria for analysis were employed for the statistics presented in Tables 4.2 (page 65) 5.1 (page 74) and 5.2 (page 77).

Since it has been shown that in some cells a functional p53 protein is required for apoptosis (Clarke et al., 1993; Lowe et al., 1993) and A2780/cp70 cells appear to contain a dysfunctional p53 protein as measured by a lack of radiation-induced G1 arrest, it is possible that the reduced ability of A2780/cp70 cells to engage apoptosis in response to ionising radiation is as a result of this p53 dysfunction.

Table 4.1. P values calculated using the Students *t*-test:

Irradiated A2780 v irradiated A2780/cp70

TEST	P VALUE
A2780 NT v A2780 2Gy	0.095*
A2780 NT v A2780 4Gy	0.001**
A2780 2Gy v A2780/cp70 4Gy	0.046**
A2780 4Gy v A2780/cp70 4Gy	0.003**

NT, No treatment

* Statistically significant at a level of $P<0.1$

** Statistically significant at a level of $P<0.05$

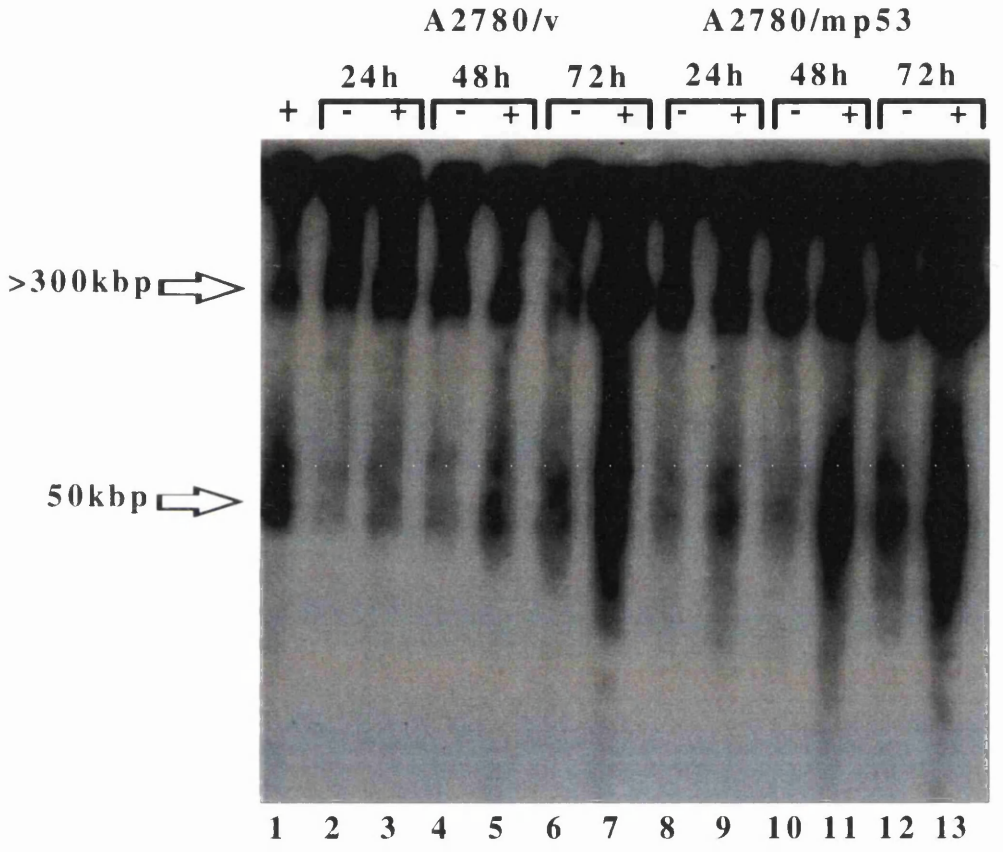
4.5 Is ionising radiation-induced apoptosis dependent on the presence of functional p53?

In order to investigate the role of functional p53 protein in the ionising radiation-induced apoptotic response I performed similar experiments to those described in sections 4.3 and 4.4 using the vector-alone and mutant p53 transfectant cell lines.

4.5.1 Field Inversion Gel Electrophoresis of irradiated A2780/v and A2780/mp53 cells

Figure 4.6 shows the pattern of DNA fragmentation observed in A2780/v and A2780/mp53 cells after 4Gy ionising radiation. At 72h after irradiation there was an increase in the amount of the larger (300-1000kbp) DNA fragments in A2780/v cells compared with unirradiated A2780/v cells (lanes 6&7). At 48h and 72h there was an increase in fragments of 50kbp in size in the irradiated cells compared with controls (lanes 4-7) much the same as was seen in irradiated A2780 cells. A2780/mp53 cells showed an increase in the appearance of 300-1000kbp DNA fragments 72h after irradiation compared with controls (lanes 12&13). The mutant p53 transfected cells also displayed an increase in the appearance of 50kbp fragments in irradiated cells compared with controls at all three time points (lanes 8-13). Thus it would appear that quite a high level of apoptosis-associated DNA fragmentation was occurring in A2780/mp53 cells, however the autoradiographic signal from the wells becomes progressively higher moving from left to right across the gel suggesting that the DNA loading was not consistent for all the wells or, much more likely, that the DNA was unevenly blotted onto the nylon membrane. Nevertheless, there was a high level of DNA fragmentation in the radioresistant cell line. It is clear that the expression of a dominant negative p53 in the wild type p53 background of A2780 cells did not completely abolish the apoptotic response.

Figure 4.6 Autoradiograph showing results of FIGE with DNA from unirradiated (-) and 4Gy irradiated (+) A2780/v (lanes 2-7) and A2780/mp53 cells (lanes 8-13). The positive control (lane 1) is the 4Gy irradiated A2780 DNA sample shown in Figure 3.2.



4.5.2 Percentage ionising radiation-induced apoptosis in A2780/v and A2780/mp53 cells

TUNEL/PI staining and analysis by flow cytometry gave a much clearer picture of the levels of apoptosis in the transfected cell lines. For the purpose of this experiment both cell lines were irradiated with an equally damaging dose of 4Gy. Figure 4.7 shows the data from four repeat experiments. Apoptosis was 3 fold higher in A2780/v than in A2780/mp53 at 24h after 4Gy ionising radiation however the level of apoptosis in both cell lines was very low at this time. No difference in apoptosis was observed at 48h after irradiation however apoptotic cells were detected in both cell lines. At 72h after irradiation apoptotic cells constituted 14% of the total A2780/v cell population compared with 4% of the A2780/mp53 cells and at 96h apoptotic cells constituted 20% of the A2780/v population and 8% of the A2780/mp53 cell population. This translates to 3.5 fold and 2.5 fold higher apoptosis in irradiated A2780/v cells compared with A2780/mp53 cells at 72h and 96h respectively. The data from the 72h and 96h time points were used to test for a statistically significant difference in apoptosis levels between the two cell lines. As shown in Table 4.2 4Gy of ionising radiation induced significantly more apoptosis in the vector alone transfectants than in the mutant p53 transfectants ($P=0.026$).

Figure 4.7 Percentage ionising radiation-induced apoptosis in A2780/v (solid lines) and A2780/mp53 cells (dotted lines) as measured by TUNEL/PI staining and FACs analysis. Error bars represent the standard error of the mean of at least three duplicate experiments.

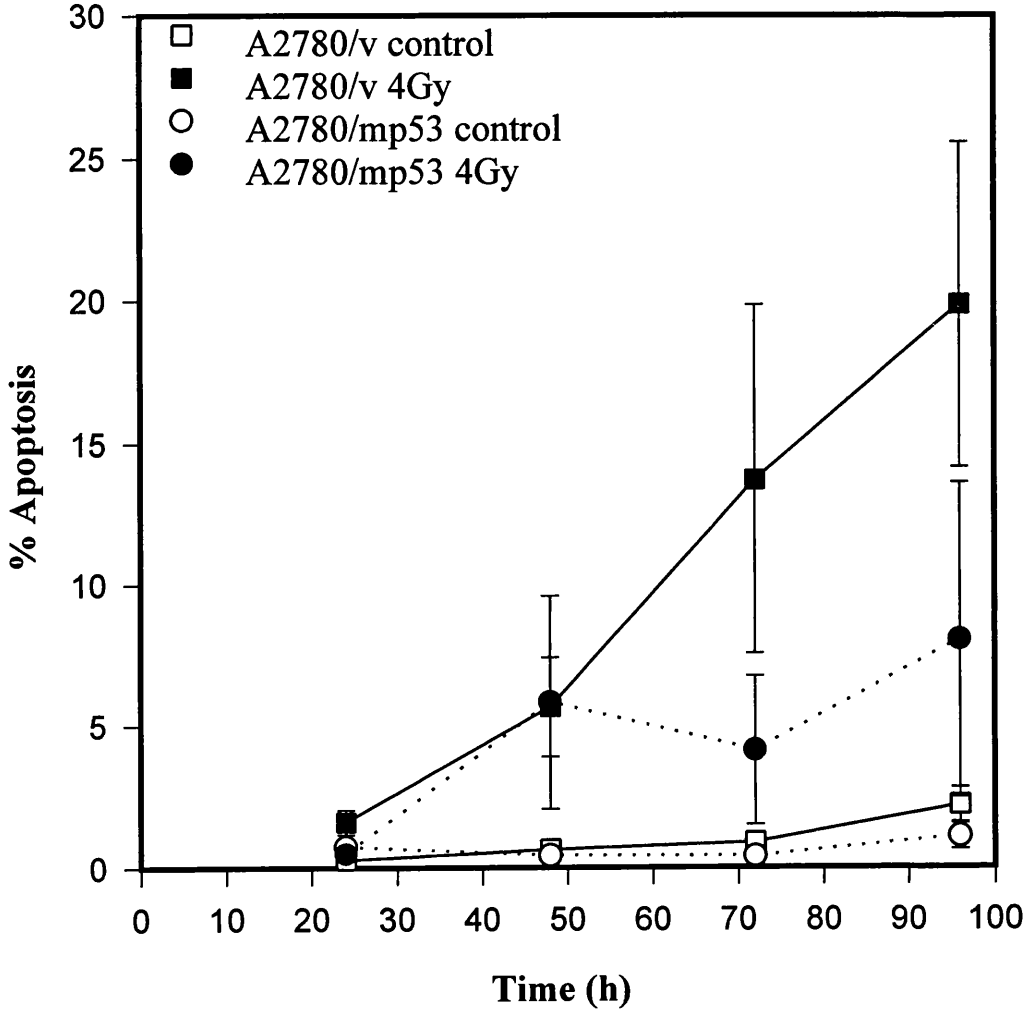


Table 4.2 P values calculated using the Students *t*-test:

Irradiated A2780/v v irradiated A2780/mp53

(See page 59 for details of statistical analysis)

TEST	P VALUE
A2780/v NT v A2780/v 4Gy	0.001**
A2780/v 4Gy v A2780/mp53 4Gy	0.026**

NT, No treatment

** Statistically significant at a level of $P < 0.05$

Thus transfection of A2780 cells with a dominant negative mutant of *TP53* not only causes an abrogation of the radiation-induced G1 arrest but also renders the cells more resistant to ionising radiation, which is manifested in a reduction in the ability of the cells to engage radiation-induced apoptosis. This demonstrates that ionising radiation-induced apoptosis in the A2780 cells is dependent at least in part on wild type p53 function.

4.6 Conclusions

Results from FIGE and TUNEL/PI staining demonstrated that A2780 cells apoptosed in response to ionising radiation. This response was most evident at 72h and 96h after irradiation. A2780/cp70 cells did not completely lose their ability to apoptose but did so to a much lesser extent than did A2780 cells. The increased apoptosis in A2780 cells compared with A2780/cp70 was seen even when the two cell lines were treated with

equitoxic doses of irradiation, suggesting that the radioresistant A2780/cp70 cells are inherently less able to engage apoptosis than are the radiosensitive A2780 cells.

Transfection of a dominant negative mutant of *TP53* (codon 143, val-ala) into A2780 cells caused a reduction in the ability of the cells to engage apoptosis at 72h and 96h after irradiation compared with cells containing wild-type p53. There was no difference in the levels of apoptosis between the two cell lines at 24h and 48h after irradiation. The apoptosis observed in A2780/mp53 cultures may be due to the fact that it retains it's wild-type p53 background whilst overexpressing a transfected dominant negative p53 protein and so may retain some transcriptional transactivation activity and an ability to undergo p53-dependent apoptosis. It is also possible that some of the p53 protein present in A2780/mp53 cells as a result of the transfection may be able to transiently or otherwise take on a wild type conformation so enabling the cells to undergo a low level of p53-dependent apoptosis. This however, would not explain the change in kinetics of the apoptotic response to ionising radiation in A2780/mp53 cells compared to A2780/v cells. It is possible though, that the change in kinetics may reflect the alteration in cellular metabolism which can result from the overexpression of an exogenous protein or transfection of exogenous DNA. The plating efficiency of A2780/v cells was comparable to that of the parental A2780 cell line. A2780/cp70 cells had a slightly higher plating efficiency than A2780 cells however A2780/mp53 cells, overexpressing the mutant p53 protein had a reduced plating efficiency compared with vector-alone transfectants (and A2780 and A2780/cp70 cells). This would suggest that, rather than the transfection of exogenous DNA, it may be the overexpression of exogenous protein that could be causing a disruption in the metabolism of A2780/mp53.

It was clear that wild-type p53 expressing A2780/v cells underwent more ionising radiation-induced apoptosis than mutant p53 expressing A2780/mp53 cells. Thus there

was a correlation between loss of p53 function, increased resistance to ionising radiation and a reduction in the ionising radiation-induced apoptotic response in A2780 cells.

CHAPTER 5

CISPLATIN-INDUCED APOPTOSIS IN HUMAN OVARIAN CELL LINES

5.1 Aims

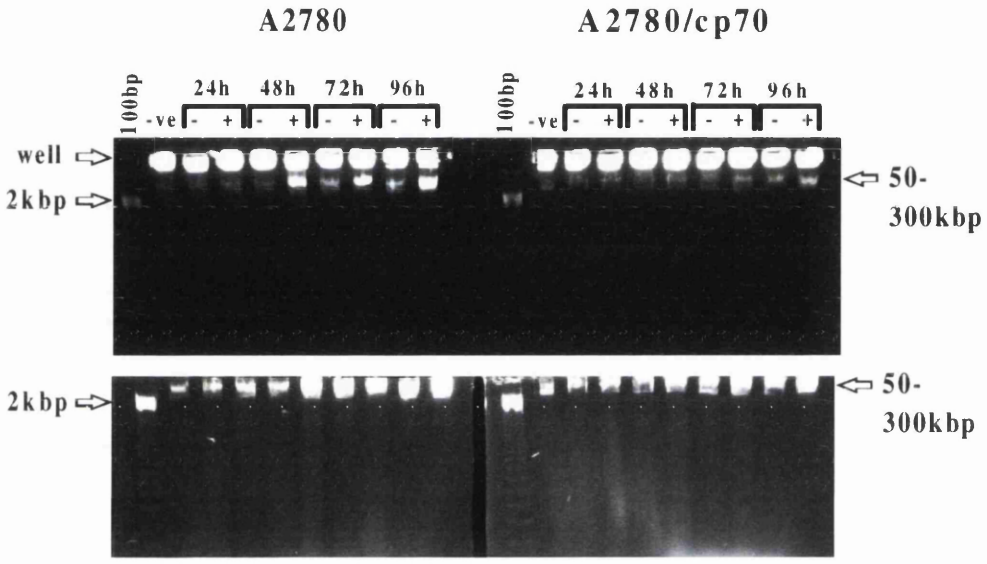
The A2780/cp70 cell line was derived from A2780 cells by multiple exposures to cisplatin up to a maximum concentration of 70μM. I assessed the apoptotic response of both cell lines to cisplatin to determine if a reduction in the ability of the cells to engage apoptosis contributes to the reduction in sensitivity to cisplatin of A2780/cp70 compared to the parental cell line. I measured cisplatin-induced apoptosis in the A2780/v and A2780/mp53 cell lines to determine the effect of overexpression of mutant p53 in the cells on their ability to apoptose.

Further cisplatin resistant derivatives of A2780 (see section 2.1.7) were used to determine the uniformity of reduced ability to engage apoptosis following acquired cisplatin resistance in multiple cell lines. A possible role for Bcl-2 in apoptosis in this cell line model was also investigated.

5.2 Lack of DNA laddering in A2780 and A2780/cp70 cell lines in response to cisplatin

Figure 5.1 shows the ethidium bromide gel of A2780 and A2780/cp70 cells treated with 40μM cisplatin, lysed in agarose and electrophoresed. The vast majority of the DNA remained in the wells and there was no detectable internucleosomal laddering in the cisplatin treated cells (see long exposure photograph, bottom panel, Figure 5.1). The short exposure photograph (top panel, Figure 5.1) revealed the presence of large sized DNA fragments which were more abundant in treated A2780 cells at 48h, 72h and 96h after cisplatin treatment compared with controls. There was also a slightly increased abundance of these large DNA fragments in A2780/cp70 cells 96h after treatment. As was the case after exposure to 4Gy ionising radiation (see Chapter 3, section 3.2.1)

Figure 5.1 Photograph of an ethidium bromide stained agarose gel showing electrophoresed A2780 and A2780/cp70 DNA after either no treatment (-) or after a 1h exposure to 40μM cisplatin (+). The upper panel shows a short exposure of the gel and includes the wells. The lower panel shows a longer exposure with the wells removed for clarity.

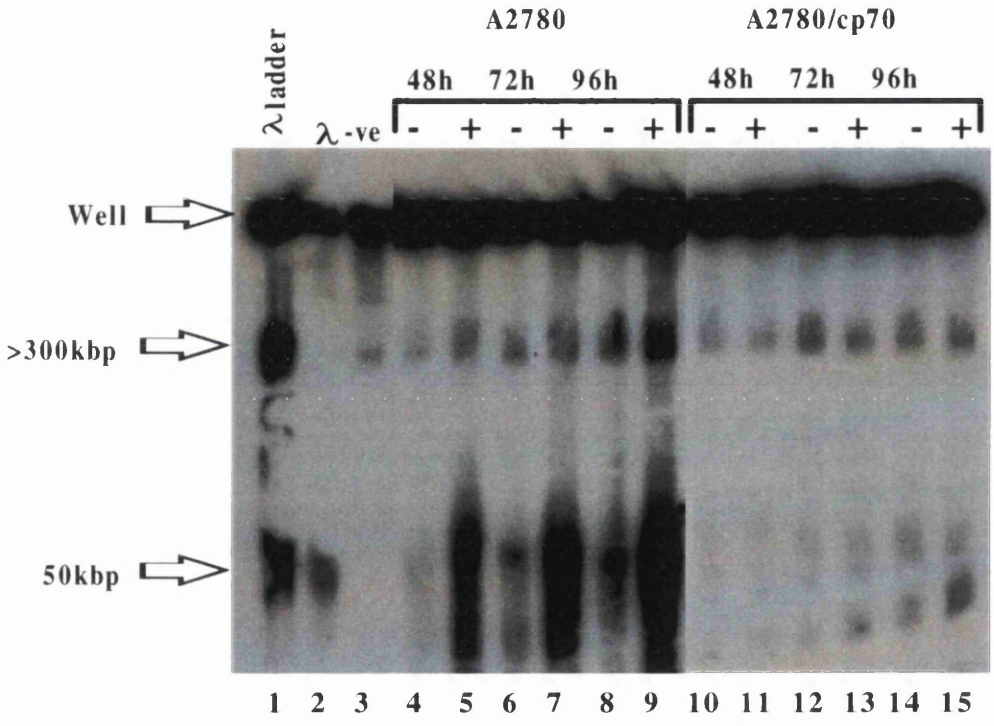


A2780 and A2780/cp70 cells did not produce internucleosomal DNA ladders after treatment with 40μM cisplatin. To further elucidate the pattern of DNA degradation in the two cells lines in response to cisplatin the same DNA preparations as used here were subjected to field inversion gel electrophoresis.

5.3 Production of 50kbp and 300-1000kbp DNA fragments in A2780 and A2780/cp70 by cisplatin

Field inversion gel electrophoresis was used to resolve the large sized DNA fragments observed in Figure 5.1. The same DNA samples as shown in Figure 5.1 were used. Figure 5.2 shows the autoradiograph of the field inversion gel. DNA damage by cisplatin (in addition to that caused by ionising radiation; see section 3.3 and section 4.3) was capable of inducing the production of the 50kbp and 300-1000kbp DNA fragments, characteristic of apoptosis in these cell lines. At all the times investigated (48h, 72h and 96h) there was more non-random DNA fragmentation in 40μM cisplatin-treated A2780 cells compared with untreated A2780 cells. Substantially more non-random DNA fragmentation was observed in cisplatin-treated A2780 cells than in cisplatin-treated A2780/cp70 cells. Indeed, no increase in DNA fragmentation was observed in A2780/cp70 cells 48h and 72h after 40μM cisplatin exposure and only a very slight increase in fragmentation compared with control A2780/cp70 cells was observed 96h after treatment.

Figure 5.2 Autoradiograph of a field inversion gel showing A2780 DNA (lanes 4-9) and A2780/cp70 DNA (lanes 10-15) after either no treatment (-) or after a 1h exposure to 40μM cisplatin (+). DNA preparations are the same as those used in Figure 5.1.



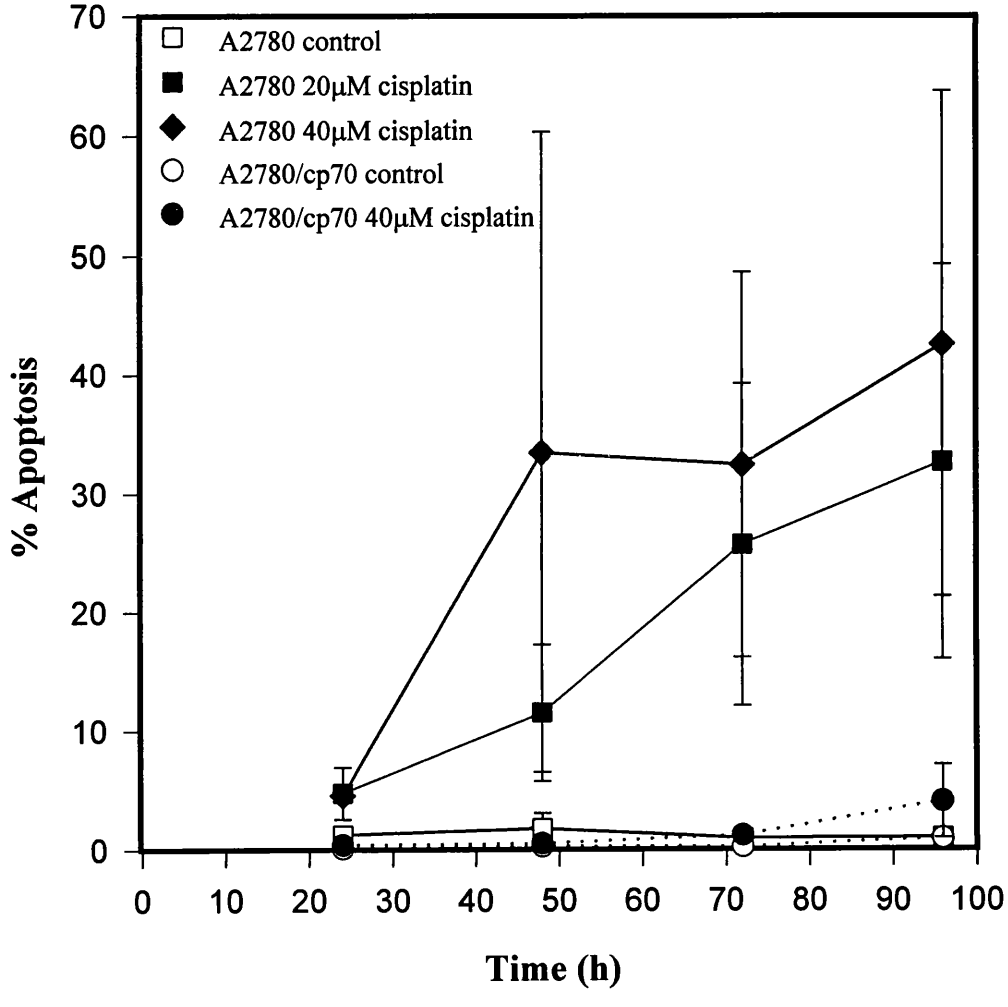
5.4 Percentage apoptosis induced by cisplatin in A2780 and A2780/cp70

Cisplatin-induced apoptosis was determined by comparing A2780 cells treated with 20μM and 40μM concentrations of the drug with 40μM cisplatin-treated A2780/cp70 cells. This allowed a comparison of the effects of the same dose (40μM cisplatin) on each of the cell lines and of the effects of doses which introduce similar levels of total cisplatin lesions in each cell line (20μM cisplatin in A2780 cells and 40μM cisplatin in A2780/cp70 cells, Johnson et al., 1994a).

Upon quantitating the percentage of A2780 cells which apoptose after cisplatin treatment it was found that there was a degree of inter-experimental variation. This variation could have been due to differences between batches of cisplatin (three different batches were used). Other possible sources of experimental variation are differences in the amount of drug presented to the cells on any one occasion, differences in the length of exposure to the drug and variation in growth medium.

It can be seen from Figure 5.3 that a concentration of 40μM cisplatin did not induce substantial levels of apoptosis in A2780/cp70 cells at the times examined. High levels of apoptosis were induced by both 20μM and 40μM cisplatin in A2780 cells (see Table 5.1 for statistical analysis). It has been shown that 20μM cisplatin introduces the same amount of platination into A2780 cells as does 40μM cisplatin into A2780/cp70 cells (Johnson et al., 1994a). As shown in Table 5.1 there was significantly more apoptosis induced in A2780 cells by 40μM cisplatin than there was in A2780/cp70 cells treated with the same concentration of the drug ($P=0.005$). The difference in apoptosis between the two cell lines treated with equally damaging concentrations of the drug was marginally statistically significant ($P=0.071$). This suggests that in A2780/cp70 cells the

Figure 5.3 Percentage cisplatin-induced apoptosis in A2780 and A2780/cp70 cells as measured by TUNEL/PI staining and FACs analysis. Error bars represent the standard error of the mean of at least three duplicate experiments.



cisplatin-DNA lesions were less capable of triggering an apoptotic response than in the parental cell line.

Table 5.1 P values calculated using the Students *t*-test:

Cisplatin treated A2780 v cisplatin treated A2780/cp70

(See page 59 for details of statistical analysis)

TEST	P VALUE
A2780 NT v A2780 20μM	0.037**
A2780 NT v A2780 40μM	0.001**
A2780 20μM v A2780/cp70 40μM	0.071*
A2780 40μM v A2780/cp70 40μM	0.005**

NT, No treatment

* Statistically significant at a level of $P<0.1$

** Statistically significant at a level of $P<0.05$

One of the three batches of cisplatin used in the above experiments induced a very high level of apoptosis in A2780 cells (about 80% at 72h and 96h) and so the same batch of cisplatin was used again to investigate the extent to which experimental variation, as opposed to cisplatin batch variation, was contributing to the different levels of apoptosis observed in A2780. The cells again showed a very high level of apoptosis (65%) but only at 96h, so it would appear that the measurement of cisplatin-induced apoptosis is

sensitive to both variations between drug batches and to the variations which inevitably occur when treating cells with a drug, such as cisplatin, which does not readily dissolve in aqueous solution.

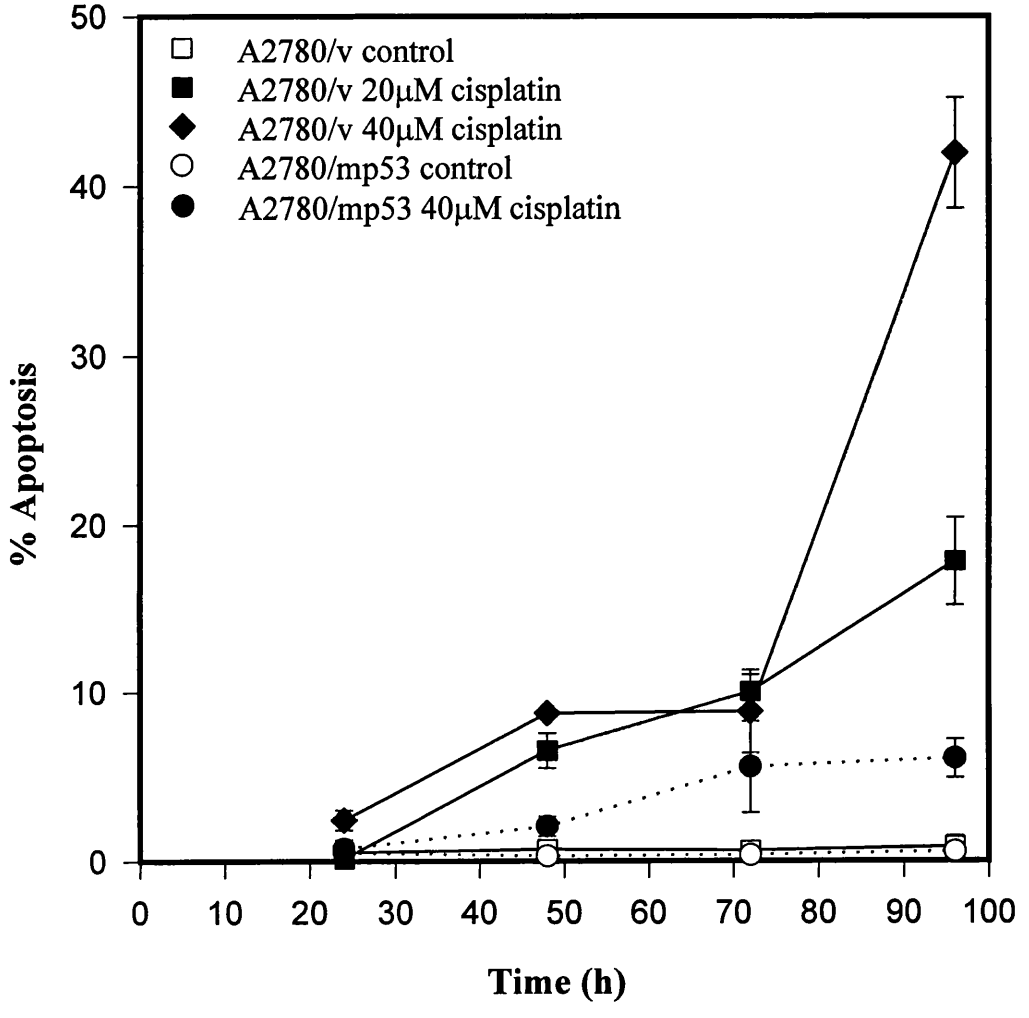
Despite the variations in absolute levels of apoptosis in A2780 cells it was clear that upon treatment of A2780 and A2780/cp70 with either equal concentrations of cisplatin or with concentrations which introduce equal amounts of DNA damage into the two cell lines more apoptosis was induced in the A2780 cell line. Only limited conclusions can be drawn on the kinetics of cisplatin-induced apoptosis. A statistically significant level of apoptosis was not observed as early as 24h. Maximal apoptosis in A2780 cells was observed at 72h after treatment in two out of four experiments and at 96h in the other two cases.

5.5 Is cisplatin-induced apoptosis dependent on functional wtp53 in A2780 cells?

A2780/v cells were treated with 20μM and 40μM cisplatin and the levels of apoptosis observed were compared with the levels of apoptosis seen in 40μM cisplatin-treated A2780/mp53 cells.

The level of apoptosis detected in control cultures at all time points was less than 1%. Cisplatin-induced apoptosis appeared to be dose dependent in A2780/v cells (Figure 5.4). A higher percentage of apoptotic cells was observed in 40μM cisplatin-treated A2780/v cell cultures than in 20μM cisplatin-treated cultures (especially at 96h after treatment). Maximal apoptosis occurred in A2780/v cultures at 96h after treatment with both concentrations of cisplatin (18% apoptosis in 20μM cisplatin-treated cultures and 42% apoptosis in 40μM cisplatin-treated cultures). The percentage of apoptotic cells

Figure 5.4 Percentage cisplatin-induced apoptosis in A2780/v and A2780/mp53 cells as measured by TUNEL/PI staining and FACs analysis. Error bars represent the standard error of the mean of at least four duplicate experiments.



observed in 40μM cisplatin-treated cultures was greater in the A2780/v cell line than in A2780/mp53 cell line at all four time points. This increased level of cisplatin-induced apoptosis in the wild-type p53 cells was marginally statistically significant (P=0.065, see Table 5.2). A similar pattern of increased cisplatin-induced apoptosis in A2780/v was observed when 20μM cisplatin-treated A2780/v cells were compared with 40μM cisplatin-treated A2780/mp53 though this trend was not statistically significant.

Table 5.2 P values calculated using the Students *t*-test:

Cisplatin treated A2780/v v cisplatin treated A2780/mp53

(See page 59 for details of statistical analysis)

Test	P value
A2780/v NT v A2780/v20μM	0.005**
A2780/v NT v A2780/v 40μM	0.008**
A2780/v 20μM v A2780/mp53 40μM	0.433
A2780/v 40μM v A2780/mp53 40μM	0.065*

NT, No treatment

* Statistically significant at a level of P<0.1

** Statistically significant at a level of P<0.05

5.6 Cisplatin-induced apoptosis in multiply selected cisplatin resistant cell lines

It is clear thus far that the acquisition of cisplatin resistance and the concomitant acquisition of ionising radiation resistance manifested in A2780/cp70 cells is, at least partly, mediated by a reduction in the ability of cells to engage apoptosis. To determine if this was a common characteristic of acquired cisplatin resistance in A2780 cells I measured the cisplatin-induced apoptotic response in nine other cell lines (A2780/mcp1-9, see Table 5.3). These lines were derived by selecting A2780 cells for resistance to multiple exposures of increasing concentrations of cisplatin (see section 2.1.7 and Anthoney et al., 1996). These cell lines were maintained, as were A2780/cp70 cells, without further selective pressure from cisplatin exposure. Eight out of the nine cells lines had substantially reduced apoptotic responses to 40μM cisplatin compared with the parental A2780 cells. In four of the lines (A2780/mcp2, 3, 4 and 8) a high fold resistance (greater than or equal to 3.5 fold more resistant to cisplatin compared with A2780 cells) correlated well with a low apoptotic response (less than 2% apoptosis 72h after 40μM cisplatin for 1h). A2780/mcp1, 5 and 6 cell lines had a lower fold resistance (between 1.5 and 2.5 fold more resistant to cisplatin than the parental cell line) and had intermediate levels of apoptosis of 4%, 8%, and 7% respectively. The majority of these cell lines had also lost p53 function as measured by lack of an ionising radiation-induced G1 cell cycle arrest. Two of the cell lines were more unusual. One (A2780/mcp9) had a very low apoptotic response of 1% and yet was only 1.5 fold more resistant than the parental line. It is possible that in this cell line, the maximal level of apoptosis was occurring at a different time. The other line (A2780/mcp7) retained a high apoptotic response (20%) whilst being 3 fold more resistant to cisplatin than A2780 cells.

Table 5.3 Apoptosis in multiply selected cisplatin resistant derivatives of A2780. Percentage apoptosis was measured 72h after 40μM cisplatin by TUNEL/PI staining and FACs analysis. Fold resistance was determined by clonogenic assay by D.A. Anthoney. G1 arrest data was also acquired by D.A. Anthoney and was determined as the percentage of cells in S phase 24h after 2Gy ionising radiation.

CELL LINE	FOLD RESISTANCE	%CISPLATIN APOPTOSIS	G1 ARREST
A2780	1	22	0.54
A2780/CP70	5	1	1.05
A2780/MCP1	1.5	4	0.94
A2780/MCP2	3.5	2	1.03
A2780/MCP3	5	1	0.98
A2780/MCP4	4	1	1.2
A2780/MCP5	2	8	0.62
A2780/MCP6	2.5	7	1
A2780/MCP7	3	20	0.64
A2780/MCP8	3.5	1	0.82
A2780/MCP9	1.5	1	0.71

The above values for cisplatin induced apoptosis were obtained after cells had been grown in culture for approximately two months (20 passages) following isolation. I also measured the apoptotic response of three of the cell lines shortly after isolation (passage 5) and again approximately 20 passages later. Shortly after isolation A2780/mcp1 and A2780/mcp5 retained a degree of the apoptotic response observed in the parental line (Table 5.4). A2780/mcp1 had already lost the p53-dependent G1 arrest in response to ionising radiation while A2780/mcp5 had retained this response. After expansion in culture their ability to engage apoptosis in response to cisplatin was severely

compromised. In addition loss of the apoptotic response in A2780/mcp5 cells correlated with loss of the p53-dependent G1 arrest. Therefore following initial selection for resistance to cisplatin there was a further pressure for selection being exerted in these two cell lines in the absence of cisplatin. Table 5.4 also shows the percentages of apoptotic A2780/mcp7 cells under the same conditions. These cells did not lose the ability to undergo apoptosis and retained the p53-dependent, ionising radiation-induced G1 arrest.

Table 5.4 Loss of apoptotic response of cisplatin resistant cells in culture. Levels of apoptosis were determined 72h after 40μM cisplatin. The data for G1 arrest is the percentage of cells in S phase 24h after 2Gy ionising radiation and was acquired by D.A. Anthoney.

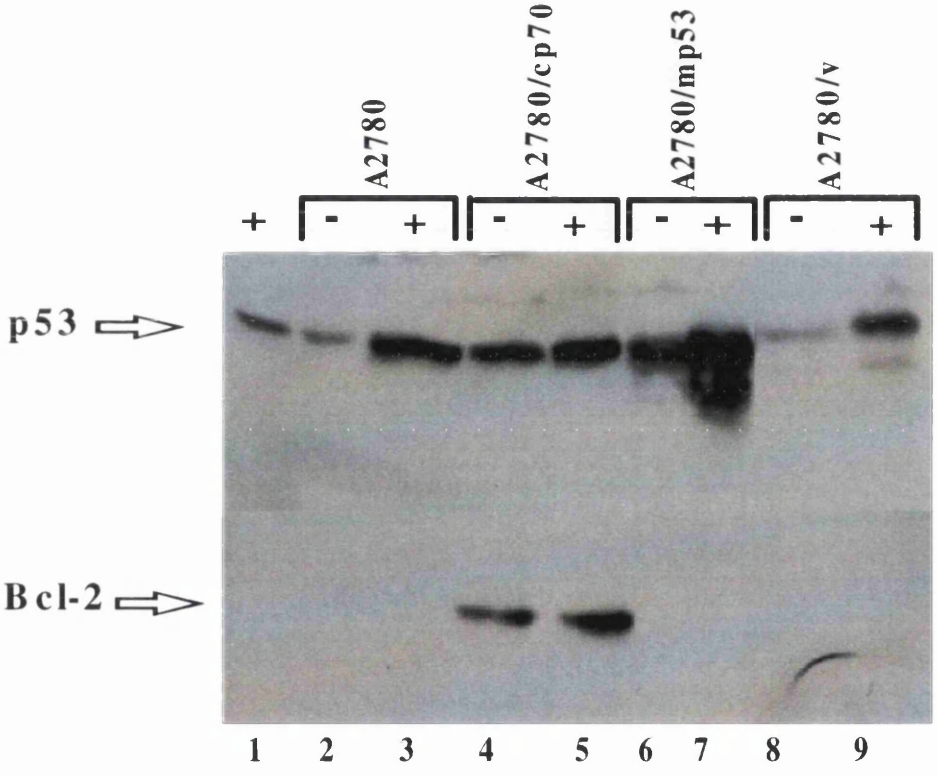
CELL LINE	PASSAGE	G1 ARREST	% APOPTOSIS
A2780	5	0.54	23
	25	0.56	22
MCP1	5	0.94	7
	25	0.84	1
MCP5	5	0.62	13
	25	1.15	4
MCP7	5	0.64	22
	25	0.69	18

5.7 Proteins levels of p53 and Bcl-2 in A2780, A2780/cp70, A2780/v and A2780/mp53

Bcl-2 has been shown to play a protective role in some cell types, in favour of cell survival (Hockenbery et al., 1990). Figure 5.5 shows the levels of p53 protein and Bcl-2 protein in whole cell extracts from the A2780 cell line and from some of its' derivatives. The anti-human p53 antibody used, cross reacts with both wild-type and mutant p53 protein. As has been shown previously (Brown et al., 1993) wild-type p53 protein accumulates in A2780 cells (compare lanes 2 and 3) and in A2780/cp70 cells (compare lanes 4 and 5) 24h after 4Gy irradiation. A2780/v transfectant cells behaved similarly to the parental cell line (lanes 8 and 9). A2780/mp53 cells contained a constitutively higher level of p53 protein as would be expected from a cell line expressing protein from a transfected mutant *TP53* gene. Following irradiation of A2780/mp53 cells the p53 protein accumulates, probably due to stabilisation of the endogenous wild-type p53.

Bcl-2 protein was only observed in extracts from A2780/cp70 cells and was not detected in the parental line or in its' transfected derivatives. This lack of detection may indicate that the Bcl-2 protein is highly unstable in A2780 but is more likely to be due to limited sensitivity of the experimental protocol. Bcl-2 protein has been found to be expressed in A2780 cells (N. Jones, personal communication). Introduction of mutant p53 (codon 143, val to ala) had no detectable effect on the expression of Bcl-2 in A2780 cells. Furthermore, induction of DNA damage did not appear to cause an accumulation of Bcl-2 in A2780/cp70 cells. If Bcl-2 does accumulate in response to DNA damage it may only be apparent at a time at which apoptosis is induced in A2780 cells and so a 72h time point may be more appropriate however no Bcl-2 accumulation was observed in these cells in response to cisplatin (N. Jones, personal communication).

Figure 5.5 SDS-PAGE gel of protein from 4Gy irradiated (+) and unirradiated (-) A2780 (lanes 2 and 3), A2780/cp70 (lanes 4 and 5), A2780/mp53 (lanes 6 and 7) and A2780/v (lanes 8 and 9). The gel was Western blotted and probed with anti-human p53 and anti-human Bcl-2 antibodies. Positive control is a protein preparation from A2780 cells known to cross react with the anti-human p53 antibody. Protein sizes were confirmed using pre-stained molecular weight markers.



5.8 Conclusions

A2780 cells were capable of undergoing apoptosis in response to cisplatin treatment as demonstrated by detection of non-random DNA fragmentation. As was observed in response to ionising radiation, these cells did not produce the DNA ladder on agarose gels following cisplatin treatment. This may indicate that the degradation of DNA to 50kbp and 300-1000kbp fragments and the lack of DNA laddering is not agent specific. A2780 cells did not require the degradation of DNA to internucleosomal fragments in order to die by apoptosis. Indeed they may be incapable of either producing these fragments or of surviving long enough after the initial apoptosis signal for further DNA degradation to occur.

It was clear from field inversion gel electrophoresis that A2780/cp70 cells underwent very little apoptosis as evidenced by the extremely low level of DNA degradation occurring in these cells. There was an unavoidable high degree of interexperimental variation in the quantitation of cisplatin-induced apoptosis. Nonetheless during any one experiment a lower degree of apoptosis was always observed in A2780/cp70 cells as compared with A2780 cells and the increased levels of apoptosis in the parental cell line were statistically significant albeit marginally when comparing equally damaging concentrations of cisplatin.

A slightly reduced level of apoptosis was observed in A2780/v cells as compared with the parental cell line. This may indicate that the transfection of the vector alone was capable of rendering the cells less sensitive to cisplatin-induced apoptosis at the time points examined. The level of cisplatin-induced apoptosis in A2780/mp53 cells however, was even further reduced, indicating a role for p53 in the sensitivity of A2780 cells to cisplatin and a role for dysfunctional, mutant p53 in the acquisition of cisplatin resistance in this cell line. A role for the loss of p53 function in the acquisition of cisplatin

resistance was supported by the reduced apoptotic response of eight out of nine cisplatin resistant derivative lines of A2780 selected by multiple exposures of A2780 cells to increasing concentrations of cisplatin.

The measurement of apoptosis in the multiple step-selected cisplatin-resistant lines also revealed a possible means for determining the sequence of events which led from cisplatin exposure to cisplatin resistance. A2780/mcp1 and A2780/mcp5 lost their apoptotic response with expansion in culture. The other multiply selected cisplatin resistant lines (with the exception of A2780/mcp7) all displayed similarly low levels of cisplatin-induced apoptosis both shortly after isolation and following expansion in culture. Presumably these cell lines had lost their apoptotic response at a time prior to A2780/mcp1 and A2780/mcp5. Cell stocks of all of these lines were frozen at each stage of cisplatin exposure (D.A. Anthoney, personal communication). These cells could be examined for their responses to cisplatin-induced apoptosis in conjunction with other cellular events (such as loss of p53 function) in order to elucidate the chain of events leading to cisplatin resistance in this cell line model.

As alluded to above, A2780/mcp7 did not lose it's apoptotic response to cisplatin 72h after exposure to the drug yet was resistant to cisplatin as measured by clonogenic assay. This suggests a different mechanism for cisplatin resistance in this derivative of A2780 to that operating in A2780/cp70 and in the other multiple step-selected cisplatin resistant cell lines.

The expression of one protein that could possibly be involved in loss of apoptosis in A2780/cp70 and A2780/mp53 was studied. The Bcl-2 protein which acts as a protection against apoptosis in some cell lines was found to be more highly expressed in A2780/cp70 cells than in A2780 cells. This protein may be involved in the reduced ability of A2780/cp70 cells to engage apoptosis however the cellular changes responsible for

this increase in Bcl-2 expression are unknown. Ionising radiation resistance induced by the transfection of a mutant *TP53* (codon 143, val to ala) did not involve an increase in Bcl-2 expression.

CHAPTER 6

DISCUSSION

6.1 Summary of results

6.1.1 Development of assays to detect and quantitate apoptosis

Ionising radiation and cisplatin were used as DNA damaging agents to induce apoptosis within A2780 cell cultures in order to establish methodologies for the detection and quantitation of apoptosis. The most accurate and objective method for detecting apoptosis in A2780 cells was found to be field inversion gel electrophoresis of DNA. The DNA was obtained by lysing cells in pellets of low gelling temperature agarose. Apoptosis was quantitated in cell populations using fluorescent end-labelling of DNA fragments by TUNEL and propidium iodide staining. TUNEL utilises terminal DNA transferase to conjugate biotinylated nucleotides to the 3′-OH ends of DNA which can then be detected by binding of streptavidin-linked fluorescein. The total cellular DNA is stained with propidium iodide to allow visualisation of the cell cycle phases by FACS analysis. These cells, viewed by confocal microscopy, displayed cellular morphology characteristic of apoptotic cells: dense chromatin, pyknotic nuclei around the cell margin, apoptotic bodies budding from cells. In addition, cells displaying apoptotic morphology were also those which displayed increased green fluorescence due to TUNEL staining.

6.1.2 Apoptosis in A2780 cells

A2780 cells engaged apoptosis in response to DNA damage generated by both ionising radiation and cisplatin. The DNA of apoptotic A2780 cells underwent a process of non-random fragmentation, ubiquitous to apoptosis in other cell types (Oberhammer et al., 1993). The DNA degrades in response to the engagement of a programmed cell death pathway that controls the death of cells subjected to lethal doses of DNA

damaging agents (Kerr et al., 1994). DNA fragments of two sizes were produced in association with apoptosis in A2780 cells; one of 50kbp and one of 300-1000kbp. The DNA did not fragment further to produce the DNA ladder on agarose gels subjected to electrophoresis. As mentioned above, apoptotic A2780 cells stained by TUNEL/PI displayed characteristic apoptotic morphology. These same TUNEL/PI-stained cells were analysed by flow cytometry. Maximal apoptosis occurred at either 72h or 96h after cisplatin treatment. Similar kinetics of apoptosis were observed after ionising radiation treatment.

6.1.3 Apoptosis in cisplatin resistant derivatives of A2780

Ionising radiation-induced apoptosis was reduced in the A2780/cp70 cell line compared to A2780 cells. Cisplatin-induced apoptosis was reduced in nine out of ten cisplatin resistant cell lines derived *in vitro* from A2780 cells. A2780/mcp7, which was 3-fold cisplatin resistant compared to the parental line, retained the ability to apoptose in response to cisplatin. This cell line also retained the p53-dependent, DNA damage-induced G1 arrest. These observations point to the acquisition of DNA-damage reistance by a mechanism other than loss of p53 function.

A higher level of Bcl-2 was observed in A2780/cp70 cells and this may contribute to the lack of apoptosis seen in this cell line however further analysis of the expression of other Bcl-2 and Bax family members would be required to elucidate the role of Bcl-2 in the loss of apoptosis in this cell line model. It is likely that upstream events may feed into the Bcl-2 pathway in order to effect the loss of apoptotic response in these cisplatin resistant derivatives of A2780 cells.

6.1.4 The role of p53 in the apoptotic responses of A2780 cells

The role of p53 in ionising radiation- and cisplatin-induced apoptosis of A2780 cells was investigated using a mutant *TP53* (codon 143, val to ala)-transfected cell line, A2780/mp53. The control cell line for these experiments was a vector -transfected cell line, A2780/v. Transfection of the vector alone was found to cause a decrease in the level of apoptosis observed after treatment with ionising radiation however transfection with mutant p53 (codon 143, val to ala) precipitated a further decrease in the level of apoptosis observed. The level of cisplatin-induced apoptosis was found to vary between experiments however A2780/v cells did not have a consistently reduced apoptotic response as compared with the A2780 parental cell line. After equal doses of cisplatin A2780/mp53 cells underwent less apoptosis than A2780/v. Taken together, these results indicate a role for functional p53 in the ionising radiation- and cisplatin-induced apoptotic responses of A2780 cells.

6.2 Mechanisms of acquired resistance in A2780 cells

It would appear that p53 is involved in the cellular signalling leading from detection of DNA damage to the engagement of a programmed cell death pathway in A2780 cells. It has been demonstrated for other cell types, that wild type p53 is necessary for ionising radiation-induced apoptosis (Clarke et al., 1993; Lowe et al., 1993). In addition, cells lacking endogenous wild type p53 do not arrest in G1 phase after ionising radiation treatment (Kuerbitz et al., 1992). Wild-type p53 protein accumulates in A2780 cells after cisplatin (Brown et al., 1993) or ionising radiation treatment (McIlwrath et al., 1994). A2780 cells arrest in G1 phase and in G2 phase after treatment with ionising radiation and cisplatin (Brown et al., 1993). The G1 phase arrest in response to these agents and the sensitivity of the cells to them are dependent on wild-

type p53 function (Vasey et al., 1996; McIlwrath et al., 1994). The acquisition of cisplatin resistance by A2780 cells also results in decreased expression of the p53-responsive gene, *WAF1/CIP1* (Anthoney et al., 1996). A2780/cp70 cells accumulate p53 protein in response to DNA damage but it does not function to produce an upregulation of $p21^{WAF1/CIP1}$ mRNA and consequently does not induce a G1 phase cell cycle arrest. This lack of $p21^{WAF1/CIP1}$ induction although observed in resistant cell lines may not be causative for loss of apoptosis. It has been shown that $p21^{WAF1/CIP1}$ is dispensible for oncogene-induced apoptosis in mouse embryo fibroblasts (Attardi et al., 1996).

The most direct effectors of apoptosis discovered to date appear to be the ced-3/ICE-like proteases or caspases. Currently ten of these proteins have been described, caspase-1 through caspase-10 (Martins and Earnshaw, 1997). *In vitro* studies have shown that a protease cascade involving the caspases acts to transduce apoptotic signals. As such, caspases are thought of as the 'executioners' of cell death as opposed to proteins such as p53 and the Bcl-2/Bax family members which appear to modulate the 'committment' of a cell to becoming apoptotic. Overexpression of Bcl-2 or Bcl-x_L is capable of preventing the activation of the caspase cascade and inhibits hypoxia-induced apoptosis (Shimizu et al., 1996). Although A2780/cp70 was found to express more Bcl-2 than the parental cell line it would be necessary to know more about the expression of other Bcl-2/Bax family members in these cell lines before drawing any conclusions as to the possible influence of Bcl-2 on downstream events such as caspase activation.

Increased Bcl-2 protein was not observed in A2780/cp70 following ionising radiation treatment and has not been seen following treatment with cisplatin or Taxol (N. Jones, personal communication). The levels of three other Bcl-2 related proteins were also investigated in the A2780 and A2780/cp70 cell lines: Bcl-x_L, Bax and Bak. Bcl-x_L functions in a similar manner to Bcl-2 while Bax and Bak are pro-apoptotic proteins.

Bcl-x_L was expressed at a higher level in A2780/cp70 cells than in the parental cell line and did not change after drug treatment. A 21kDa form of Bax appeared after cisplatin treatment in A2780 cells. This protein has been shown to promote apoptosis and it has been suggested that the ratio of this form of Bax to Bcl-2 may be an important modulator of apoptosis (Oltvai et al., 1993). Bak was expressed at a higher level in cisplatin-resistant cells and was also induced by cisplatin treatment.

Nine out of ten of the cisplatin resistant derivative cell lines of A2780 have reduced constituent levels of *WAF1/CIP1* mRNA and a reduction in the DNA damage-induced G1 arrest. These lines also have a mutator phenotype (Table 6.1, summarised from Anthoney et al., 1996). It is these same cells that show a reduction in cisplatin-induced apoptosis (see section 5.6). A2780/mcp7 which was the only cisplatin resistant line not to lose its apoptotic response is also the only line that does not display the microsatellite instability phenotype. It is clear that the resistance mechanism in this cell line differs from that in the remainder of the lines examined in this study.

It is possible that acquired cisplatin resistance in A2780 cells arises due to the selection of cells with a mutator phenotype (RER^+ phenotype). The RER^+ phenotype is associated with a loss of mismatch repair. The RER^+ phenotype observed in the panel of cisplatin resistant lines used in this study is associated with the loss of the mismatch repair protein hMLH1 (Brown et al., 1997). MLH1 complexes with PMS2 and the MSH2/MSH6 complex to recognise and repair single base mispairs in DNA. These repair proteins have been found to bind with high affinity to a 1,2 diguanyl platinum intrastrand crosslink opposite to a single G:T mispair (Drummond et al., 1996). It is possible that the loss of hMLH1 leads to loss of DNA damage-induced apoptosis.

Table 6.1 Characteristics of cisplatin resistant A2780 cell lines. (summarised from Anthoney et al., 1996). Fold resistance, % cisplatin apoptosis and G1 arrest were determined as described in Chapter 5. RER status (Replication ERror, mutator phenotype) was determined by detection of microsatellite mutations and *WAF1/CIP1* mRNA levels were determined by Northern blot analysis using a *WAF1/CIP1* cDNA probe standardised against a *GAPDH* probe as described (Anthoney et al., 1996).

Cell line	Fold resistance	RER status	% cisplatin apoptosis	G1 arrest	Relative WAF1/CIP1 mRNA level
A2780	1	-	22	++	1
A2780/CP70	5	+	1	-	0.29
A2780/MCP1	1.5	+	4	-	0.52
A2780/MCP2	3.5	+	2	-	0.55
A2780/MCP3	5	+	1	-	0.32
A2780/MCP4	4	+	1	-	0.58
A2780/MCP5	2	+	8	+	0.33
A2780/MCP6	2.5	+	7	-	0.49
A2780/MCP7	3	-	20	+	0.95
A2780/MCP8	3.5	+	1	+/-	0.66
A2780/MCP9	1.5	+	1	+	0.56

How this signalling mechanism may interact with p53 is unclear. The defects in either p53 function or in mismatch repair suggest that the two mechanisms may operate as part of the same pathway in normal cells. The observations in A2780/mcp7 and A2780/mcp8 are anomalous with the rest of the data. In the case of A2780/mcp7, MLH1 expression is lost while p53 function remains intact. A2780/mcp8 cells retain MLH1 expression and mismatch repair activity (J. Drummond, personal communication) but display microsatellite instability and have lost the p53-dependent apoptotic response. They do, however retain a residual DNA damage-induced G1 arrest. It could be envisaged that following cisplatin-induced DNA damage, a signalling cascade is set in

action which encompasses recognition of complex lesions by mismatch repair proteins, a p53-dependent appraisal of the extent of the damage precipitating a decision to live or die and a subsequent signal to either cell cycle arrest and repair or to enter a programmed cell death pathway leading to apoptosis.

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PUBLICATIONS ARISING FROM THIS THESIS

Anthoney, D.A., McIlwrath, A.J., Gallagher, W.M., Edlin, A.R.M., Brown, R. (1996) Microsatellite Instability, Apoptosis and Loss of p53 Function in Drug-resistant Tumor Cells. *Cancer Res.* **56** 1374-1381.

Two further manuscripts containing aspects of the work covered in this thesis are also in preparation.