ANTI-APOPTOTIC PROTEINS

AND

CHOLANGIOCARCINOMA

Thesis submitted to the University of London for The Degree of Master in Surgery

2003

By

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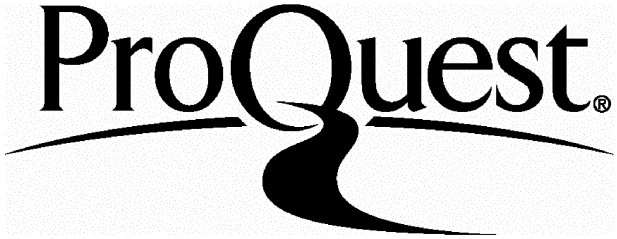

ABSTRACT

Cholangiocarcinoma is refractory to treatment by chemotherapy and radiotherapy, which exert their effects on tumour cell growth mainly through the induction of apoptosis. The factors responsible for the poor response of this disease to the apoptosis inducing effect of chemotherapy and radiotherapy are unknown. Members of the Bcl-2 family of proteins play a central role as intracellular regulators of apoptosis. *In vitro* and *in vivo* studies have identified that in certain malignancies the expression by tumour cells of these mitochondrial targeting antiapoptotic proteins provide a survival advantage to these cells. The expression of these proteins by cancer cells may also reduce their response to cytotoxic therapy.

The hypothesis of this thesis is that resistance to apoptosis may be one of the factors responsible for the poor response of cholangiocarcinoma to treatment, which may be a consequence of mitochondrial targeting antiapoptotic proteins.

In the first section of the study, the expression of the antiapoptotic proteins Bcl-2, Mcl-1 and Bcl-X_L was examined in 30 resected cases of cholangiocarcinoma, and 3 human cholangiocarcinoma cell lines using immunohistochemical and immunofluorescent techniques. In all the cholangiocarcinoma specimens examined, Mcl-1 and Bcl-X_L proteins were co-expressed by the majority of the malignant cell population. Bcl-2 protein was not however detected in any of the specimens. This confirmed that antiapoptotic proteins are expressed by cholangiocarcinoma cells but provided no information on their biological effects.

The second section analysed the kinetics of apoptosis in human cholangiocarcinoma cells after exposure to the therapeutic agents chemotherapy, X-ray and also UV irradiation to test the hypothesis that cholangiocarcinoma cells are resistant to cytotoxic therapy induced apoptosis. Human cholangiocarcinoma cell lines were incubated with various concentrations of chemotherapy drug or exposed to various doses of radiotherapy. The apoptotic responses were then monitored over a 96 hour period post treatment and then dose response graphs constucted. Cholangiocarcinoma cells were found to be resistant *in vitro* to chemotherapy and radiotherapy induced apoptosis.

Finally, Pk11195 and diamide, drugs which target the mitochondria and functionally counteract antiapoptotic Bcl-2 proteins, were used to test the hypothesis that the inhibition of antiapoptotic proteins can increase the sensivity of cholangiocarcinoma cells to therapy. Experiments were carried out both *in vitro* and *in vivo* (xenografts on SCID/NOD mice). This study confirmed that in the presence of the Bcl-2 antagonists cholangiocarcinoma cell apoptosis was increased following chemotherapy and radiotherapy. This demonstrates for the first time an association between the expression of antiapoptotic proteins Bcl-X_L and Mcl-1 and the susceptibility of cholangiocarcinoma cells to apoptosis.

The work contained in this thesis demonstrates that human cholangiocarcinoma cells express the antiapoptotic proteins Mcl-1 and Bcl-X_L and are resistant to chemotherapy and radiotherapy induced apoptosis. Antagonising the function of these proteins increases the sensitivity to both chemotherapy and radiotherapy in both cell cultures and an animal model.

Inhibitors of the antiapoptotic proteins should be further investigated for their use in conjunction with conventional cytotoxic therapy for the treatment of CCA and may be of value in the treatment of other cancers.

Chapter 2

Material and methods

Chapter 3

Antiapoptotic and Multidrug (MDR) protein P-Glycoprotein (p-gp) Expression in Human Cholangiocarcinoma

Chapter 4

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Overview and Discussion

Bibliography

ACKNOWLEDGMENTS

This work was only made possible by the help of others. I would first like to express my sincere thanks to Professors Brian Davidson and Finbarr Cotter for their guidance and support during the entire period over which the research was conducted. The commitment they showed was absolute and will always be remembered.

I am most grateful to my colleagues Dr Dean Fennell, Dr Stan Wickremasinghe, Dr Alastair Deery for their contributions and advice.

Special thanks goes to Mr John Auld, Mrs Julie Boxter and all the staff of the histopathology and cytology departments at the Royal Free Hosptital for the role they played in the preparation of the slides and the immunohistochemistry.

I am most grateful to Miss Maggie Corbo for participation in the animal experiments performed.

Lastly I will like to thank my wife Helena for her endless support and encouragement.

STATEMENT OF ORIGINALITY

Unless otherwise stated, I, with the assistance of the other acknowledged above performed all the work described in this thesis.

ETHICAL CONSIDERATIONS

The animal experiments were conducted with full adherence to the home office regulation under the supervision of Professor Finbarr Cotter the licence holder.

PUBLICATIONS

Okaro, A. C., Deery, A. R., Hutchins, R. R., & Davidson, B. R.

"The expression of antiapoptotic proteins Bcl-2, Bcl-X_L, and Mcl-1 in benign, dysplastic, and malignant biliary epithelium",

J.Clin.Pathol. 2001, vol. 54,no. 12, pp. 927-932.

Okaro, A. C, Fennell, D.A, Corbo, M, Davidson, B.R, Cotter, F.E

"Pk11195, a mitochondrial benzodiazepine receptor antagonist reduces apoptosis threshold in Bcl-X_L and Mcl-1 expressing human cholangiocarcinoma cells"

Gut, 2002. vol. 51,no. 4, pp. 556-561

PRESENTATIONS

Glutathione Depleting Agent BSO Radiosensitizes Cholangiocarcinoma cells *in vitro* to Apoptosis

Okaro A C, Cotter F A, Davidson B R

Oral presentation

British Society of Gastroenterology, International convention centre, Birmingham. 21-23 March 2000. **(GUT vol 46 suppl 11 April 2000 – abstr)**

10th Congress of the European Society of Surgical Oncology (ESSO), Groninen, Netherlands. 5-8 April 2000. **(European Journal of Surgical Oncology, April 2000 – abstr)**

4th World Congress, International Hepato-Pancreato-Biliary Association (IHPBA), Brisbane. Australia. 28 May- 1 June 2000 **(HPB vol 2 no 2 2000- abstr)**

Poster presentation

British Cancer Research Meeting 2000, Brighton, UK. 9th-12th July 2000

The Mitochondrial Benzodiazepine antagonist, Pk11195 overcomes Apoptosis Resistance in Cholangiocarcinoma cells *in vivo*

Okaro A C, Fennell D A, Cotter F A, Davidson B R

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Cholangiocarcinoma cells Express High levels of Antiapoptotic proteins Bcl-X_L and Mcl-1, but no Bcl-2- an immunohistiochemical study

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Glutathione Depleting Agent BSO Radiosensitizes Cholangiocarcinoma cells in vitro to Apoptosis

Okaro A C, Cotter F A, Davidson B R

Pk11195, The Bcl-2 antagonist lowers the Apoptosis Threshold in Cholangiocarcinoma cells Following UV Irradiation and Oxidative Stress

Okaro A C, Fennell D A, Cotter F A, Davidson B R

Oral presentation

British Association of Surgical Oncology (BASO), Royal College of Surgeons of England. 27-28 November 2000

Poster presentation

United European Gastroenterology Week (UEGW) 2000. Brussels, Belgium. 13-15 November 2000.

List of Abbreviations

ABC	Avidin-biotin complex
ADP	Adenine diphosphate
ATP	Adenine triphosphate
AEC	Three-Amino-9-Ethylcarbazole
AIP	Apoptosis initiating protein
APAAP	Alkaline phosphatase-antialkaline phosphatase
APBDJ	Anomalous Pancreatic-bile duct junction
APES	3-Amionpropyl-Triethixy-Saline
ANT	Adenonucleotide translocator
CARD	Caspase recruitment domain
CCA	Cholangiocarcinoma
CN	Four-chloro-1-Naphtol
DAB	Three, 3'-Diaminobenzidine tetrahydrochloride
DD	Death domain
DED	Death effector domain
DFF	DNA Fragmentation factor
DMEM	Dulbecco's modified essential medium
DMSO	Dimethyl-sulphoximide
DT	Diphtheria Toxin
EABA	Endogenous biotin
ECACC	European collection of cell cultures
ERCP	Endoscopic retrograde cholangiopancreatography
EUS	Endoscopic Ultrasound
FCS	Foetal calf serum
FITC	Fluorescein isothiocynate
FSC	Forward scatter
GAG	Glucose oxidase-antiglucose oxidase
H&E	Haematoxylin and eosin
ICE	Interlukin converting enzyme
LAB	Labelled avidin-biotin
MDP	Multi-drug resistance protein
MDR	Multi-drug resistance receptor
MEM	Minimum essential medium
MFI	Mean fluorescent intensity
MPT	Mitochondrial permeability transition
PAP	Peroxidase-antiperoxidase
PARP	Poly-ADP-ribose polymerase
PBD	Percutaneous biliary drain
PBS	Phosphate buffered saline
PCR	Polymerised chain reaction
PDT	Photodynamic Therapy
PPIX	Protoporphyrin IX
PT	Permeability transition
PTC	Percutaneous transhepatic cholangiography
PTD	Photodynamic therapy
PTPC	Permeability transition pore complex
ROS	Reactive oxygen species
SSC	Side scatter
TNF	Tumour necrosis factor
VDAC	Voltage dependent anionic channel
VP16	Etoposide

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INTRODUCTION

The clinical efficacy of the cytotoxic effects of chemotherapy and radiotherapy is limited by inherent or acquired mechanisms of resistance within tumour cells, preventing the successful treatment of a wide range of malignancies. Despite the availability of an increasing number of chemotherapeutic agents and advances in radiation technology used in the treatment of solid tumors, long term survival has not changed significantly in the last 20 years. Many cancers remain incurable. Bile duct cancer or cholangiocarcinoma is one example, where surgery, chemotherapy and radiotherapy have had minimal impact on overall survival. The development of new agents that have the ability to target the cellular components and reverse the mechanisms that prevent tumour cells from undergoing cytotoxicity induced apoptosis, may provide a novel approach to cancer treatment. However, the mechanisms and pathways of apoptosis resistance need to be identified.

The last decade has witnessed unprecedented advances in the study and understanding of apoptosis, the process by which chemotherapy and radiotherapy are known to kill cancer cells. Following damage to vital cellular components such as DNA, apoptosis is triggered, and is a fundamental mechanism of action of cytotoxic therapies. Apoptosis or programmed cell death, first described in 1972 by Wyllie *et al*, is an evolutionary conserved and tightly regulated process of cell death. The Bcl-2 family of genes are important regulators of this process, with members of this family either promoting (pro-apoptotic) or inhibiting (anti-apoptotic) apoptosis. Bcl-2 protein, the prototype of this family was first identified in follicular Lymphoma. However, the expression of the protein Bcl-2 is by no

means restricted to cases of lymphoma. A wide variety of solid and haematological malignancies express Bcl-2 and other apoptosis regulating proteins. In certain tumours where the levels of expression of Bcl-2 or Bcl-X_L are high, tumour cells are resistant to chemotherapy and radiotherapy, with an overall poorer outcome. Cholangiocarcinoma has been shown to express the Bcl-2 protein, but most studies have involved the analysis of a small number of cases, making interpretation of results difficult. In addition, the detection of protein in paraffin embedded samples provides no information into the cellular function of these proteins.

This thesis has tested the hypothesis that the poor response of cholangiocarcinoma to cytotoxic therapy may be a result of the expression of antiapoptotic proteins by cholangiocarcinoma cells.

The first part of this study focused on re-examining the expression of antiapoptotic proteins Bcl-2, Mcl-1 and Bcl-X_L in cholangiocarcinoma in tissue samples from 30 resected cases of cholangiocarcinoma along with three human cholangiocarcinoma cell lines. Subsequent experiments tested the hypothesis that resistance to apoptosis may be secondary to the expression of anti-apoptotic proteins in cholangiocarcinoma.

CHAPTER 1

1.1 Cholangiocarcinoma

1.1.1 History

Bile duct carcinoma has been recognised for over a century. Musser in 1889 reported 18 cases of primary extrahepatic bile duct carcinoma (Musser 1889). In 1940, the first large review (306 cases) of extrahepatic bile duct cancer was published by Stewart *et al.* (Stewart 1940). Twenty-one years later, Sako and co-workers reviewed the world literature from 1935 to 1954, finding an additional 570 cases of extrahepatic bile duct cancer (Sako 1957). In contrast to these reports of extrahepatic cases, descriptions of intrahepatic cancers are more recent (Helling 1994). Altemeier *et al.* in 1957 reported on three cases of primary adenocarcinoma of the major bile duct. These tumours would now be considered hilar cholangiocarcinoma (Altemeier 1957). In that same year Kuwayti and colleagues reported several additional patients (Kuwayti 1957), and 1 year later three additional patients were reported (Thorbjarnarson 1958). In 1965, Klatskin reported 13 patients with cancer of the hepatic duct bifurcation (Klastin 1965). This report stimulated interest in this uncommon malignancy and led to the term 'Klatskin tumour'.

The last twenty-five years has seen many innovations in the imaging, surgical resection and non-operative management of these patients.

1.1.2 Incidence

Cholangiocarcinoma has a worldwide distribution that accounts for about 10-15% of all cases of primary hepatobiliary malignancy. The true incidence of cholangiocarcinoma is dependent upon all patients being correctly diagnosed and reported. It is highest where the possibility of existence is suspected and where investigative interest before and at surgery is intense. Many cases have been missed simply because the possibility of cholangiocarcinoma was not considered (Ingis & Farmer 1975;Legge & Carlson 1972;Whelton et al. 1969). In many parts of the world, financial considerations affect diagnosis, investigation and treatment. The highest incidence reported comes from centres in Great Britain, the United States, Australia and South Africa, which specialise in biliary surgery. The highest actual incidence is in Far East Asia where parasitic infestation with the liver fluke *Clonorchis sinensis* is widespread (Viranuvatti & Stitnimankarn 1972)

Incidence may be expressed in several ways: operative and autopsy figures have been reported, also the percentage of duct tumours compared with all malignant tumours or percentage of tumours within the biliary tree. The least equivocal figures are probably those of Annual death rates, but no one method is absolutely accurate. Figures given by Longmire (1976) predict approximately 4500 new cases of bile duct cancer in the US per annum, with 4275 annual deaths. Official statistics can often be misleading. Thus the Registrar General's Cancer Mortality figures for England and Wales (1973) classify malignant tumours of the

liver and intrahepatic bile duct together, and also combine neoplasms of the gall bladder and the bile ducts with a reported incidence of 1-2/100,000 in UK.

1.1.3 Aetiology

In the majority of cases of cholangiocarcinoma, no aetiological factor can be identified, a number of conditions have been shown to be important in the development of cholangiocarcinoma. Some of the conditions associated with cholangiocarcinoma are listed in table 1. These all have in common long standing inflammation and chronic injury of the biliary epithelium (Chapman 1999). Under these conditions oxygen free radicals generated in such chronic inflammatory conditions are implicated in causing DNA damage of bile duct cells. It is speculated that growth promoting mutations such as K-ras, c-myc and c-erb-2 together with impaired apoptosis secondary to alteration in the Bcl-2 family protein expression are involved in the pathogenesis of cholangiocarcinoma (Sikora & Kapoor 1999).

Table 1 Conditions associated with the development of cholangiocarcinoma

Cystic dilatation of the bile duct
Choledochal cyst
Caroli's disease
Clonorchiasis
Primary Sclerosing Cholangitis (PSC)
Thorotrast
Hepatolithiasis

1.1.4 Clinical Presentation

The average age at diagnosis is 60-65 years, with approximately two-thirds of patients being between the ages of 50-70 (Broe & Cameron 1981a;Sons & Borchard 1987a;Yeo, Pitt, & Cameron 1990a). Patients that develop cholangiocarcinoma in association with either choledochal cyst disease, clonorchiasis, sclerosing cholangitis, ulcerative colitis, or hepatolithiasis present at younger ages. Overall, a slight male predominance has been reported, with the male to female ratio approximately 1.3 to 1. The most common finding at presentation is jaundice, which is present in over 90% of cases. Less common clinical features present in 50-60% of patients include pruritis, weight loss, anorexia, mild abdominal pain and fatigue. No finding at clinical examination is as common as jaundice. Occasionally, patients may present with hepatomegaly or a palpable gallbladder. Cholangitis is not a frequent feature at presentation, particularly prior to biliary manipulation. In contrast, cholangitis is common after endoscopic or percutenous techniques.

In cases of cholangiocarcinoma obstructing either left or right hepatic ducts, presentation is typically one of mild abdominal pain, unilobular hepatomegaly with elevated serum alkaline phosphatase or γ-glutamyltransferase, without elevation of serum bilirubin and without clinical jaundice. Such a presentation warrants full investigation including CT and cholangiography, in an effort to diagnose an intrahepatic cholangiocarcinoma early.

1.1.5 Investigations

Blood Tests

The majority of patients with cholangiocarcinoma will have elevated serum levels of total and conjugated (direct) bilirubin, as well as elevations of serum alkaline phosphatase and γ-glutamyltransferase. Typically, markers of hepatocyte injury such as alanine amino-transferase (ALT) and aspartate amino-transferase (AST) will be elevated only to a minor degree. In the vast majority of cases, other serum parameters (such as electrolytes, calcium, phosphate, cholesterol and total protein) will be normal. Occasionally patients may have mild hypoalbuminaemia, reflecting impaired hepatic protein synthesis or malnutrition. Haematological profiles in the absence of infection are usually normal. In patients with long standing jaundice there may be derangement of the prothrombin time reflecting impaired hepatic function. Serum levels of tumour markers such as carbohydrate antigen (CA19-9), carcinoembryonic antigen (CEA) or CA-125 may be normal. At present there is no reliable screening blood test diagnostic for cholangiocarcinoma.

Imaging

The goal of imaging in a patient with suspected cholangiocarcinoma is:

- The delineation of the level and degree of obstruction
- The search for metastatic disease
- The evaluation of local tumor spread, which may preclude curative resection.

An ordered use of imaging tests is typically successful in imaging the obstructing lesion and defining the biliary anatomy (Okuda, Ohto, & Tsuchiya 1988;Scharschmidt, Goldberg, & Schmid 1983). Initial radiological studies consist of either abdominal ultrasound and computerised tomography (CT). The primary tumour mass is visualised only in a minority of patients. In a comparative study of ultrasound and CT scan for hilar tumours, ultrasound detected the tumour in 21% and CT scan identified the tumour in 40% of patients (Choi et al. 1989). The imaging techniques were comparable in determining the level of obstruction when a mass was not found.

Following the documentation of bile duct dilatation by ultrasound and/or CT scan, it is imperative to define the biliary anatomy, particularly the extent of the proximal tumour involvement. Biliary anatomy can be defined cholangiographically using non-invasive means like magnetic resonance cholangio-pancreatography (MRCP) (Cieszanowski et al.;Yeh et al. 2000;Zidi et al. 2000) or invasively by endoscopic retrograde or percutenous transhepatic routes. MR has the advantage of being able not only to demonstrate the tumour position within the biliary tree (MR cholangio-pancreatography MRCP), this imaging modality can define tumour spread to adjacent vascular structures (MR angiography) and detect hepatic metastases. However, MR cannot provide a tissue diagnosis.

Fine Needle Aspiration Cytology and Biospy

Preoperative efforts to establish a tissue diagnosis in the setting of suspected cholangiocarcinoma can employ such techniques as ERCP with bile and brush cytology and endobiliary biopsy (Cope, Marinelli, & Weinstein 1988;Foutch et al. 1990;Yip et al. 1989). Bile obtained from percuteanous catheter drainage will have positive cytological features in less than 30% of cases. Preoperative endoscopic transpapillary biopsy or brush cytology techniques have reported sensitivities averaging 50% in the diagnosis of bile duct malignancy. However, obtaining a preoperative tissue diagnosis is not essential if the patient is an operative candidate. Negative results from an attempt at obtaining a preoperative tissue diagnosis are unhelpful although a positive result is useful in justifying extensive surgery.

The use of intraoperative fine needle aspiration cytology of a suspicious biliary mass has been advocated by some as an important adjunct to surgical decision making and eventual therapy for bile duct strictures and masses. However, due to the risk of tumour seeding, surgical assessment of resectability should be established prior to the biopsy being performed (Henson, Albores-Saavedra, & Corle 1992;Torzilli et al. 1999). While past results with core biopsy techniques had poor results (Lightwood, Reber, & Way 1976), later reports using intraoperative fine needle aspiration cytology of biliary masses suggest sensitivities and accuracy reaching 90% (Earnhardt et al. 1993) with low complication rates.

1.1.6 Pathology and Staging

Cholangiocarcinoma (adenocarcinoma) accounts for over 95% of all bile duct cancers and ranges from poor to well-differentiated tumours.

Cholangiocarcinoma can be categorised into papillary and nodular varieties, based upon their gross characteristics and light microscopy appearances. The pathological determination of malignancy in well-differentiated cases is often problematic due to the cellular effects of associated cholangitis, biliary obstruction, calculus disease of the biliary tree or bile duct stenting.

The location of cholangiocarcinoma within the biliary tree influences the ease of diagnosis, the choice of treatment and the eventual prognosis. The location of the primary cholangiocarcinoma can be classified into intrahepatic, perihilar and distal extrahepatic. Intrahepatic comprise 20-25% of all tumours, perihilar, which include tumours involving the hepatic duct bifurcation (Klatskin tumour) and the common hepatic ducts, account for 50-60% of cases. 20-25% are distal (de Groen et al. 1999a;O'Grady 2000)

Multiple cholangiocarcinomas are rare (Gertsch et al. 1990;Ikoma et al. 1992). Reports to date suggest that multiple tumours are more likely to occur in association with anomalous pancreatic-bile duct junction (APBDJ), PSC or choledochal cyst disease.

Autopsy studies of patients that have died from cholangiocarcinoma have indicated the presence of metastatic disease in up to 80% of cases (Sons & Borchard 1987b). Half of all patients are found to have metastatic disease within the regional lymph nodes, within the hepatic parenchyma or peritoneal surfaces

within the abdomen. In contrast to autopsy findings, the incidence of metastatic disease at initial clinical diagnosis in cholangiocarcinoma varies between 15-30%.

Cholangiocarcinoma can be staged according to the TNM classification (Sobin, Hermanek, & Hutter 1988;Spiessl 1976;Spiessl 1977). Using this classification (table1.2), stage 1 tumours are limited to the bile duct mucosa or muscle layer, stage 2 tumours extend to periductal tissue without invading lymph nodes, stage 3 tumours have regional lymph node involvement without distant metastasis while stage 4 tumours either invade adjacent structures (4a) or have distant metastasis (4b).

TNM staging of cholangiocarcinoma

T = tumour staging

T_1 = tumour limited to mucosa or muscle layer

T_2 = tumour extends to periductal tissue

T_3 = tumour extends to adjacent structures

N = nodal staging

N_0 = no evidence of regional lymph node involvement

N_1 = regional lymph node metastasis

M = metastatic staging

M_0 = no evidence of any distant metastasis

M_1 = distant metastasis (e.g. liver, lung etc.)

Stage 1 = $T_1N_0M_0$; stage 2 = T_2 N_0 M_0; stage 3 = $T_1N_1M_0$, $T_2N_1M_0$ stage 4a = T_3 any N, M_0; stage 4b = any T, any N, M_1 (from relevant references)

Assessment of Resectablilty

Patient Factors

Both patient and tumour factors must be considered when assessing the resectability of patients with cholangiocarcinoma. These factors are listed in the table below

Patient factors	Tumour factors
Age	Liver invasion
Medical condition	Colonic invasion
Cardiopulmonary status	Duodenal invasion
Liver function	Vascular invasion
Renal function	Peritoneal metastases
Nutrition	Distant metastases
Sepsis	

A specific consideration in patients with jaundice who are to undergo major surgical procedures is the cluster of physiological abnormalities associated with this condition. These abnormalities include alterations in hepatic and pancreatic function, the gastrointestinal barrier, immune function, renal function and wound healing. Obstructive jaundice affects hepatic metabolism such as hepatic mitochondrial respiratory function, hepatic protein synthesis and hepatic reticuloendothelial function (Katz et al. 1984). In addition, endotoxemia may contribute to renal, cardiac, and pulmonary insufficiency observed in patients with obstructive jaundice (Thompson et al. 1988a;Thompson et al. 1988b). Altered

cell-mediated immunity increases the risk of infection, whereas coagulation disorders make these patients prone to bleeding problems.

A number of different systems have been proposed to assess the patient-related factors in predicting mortality in patients with obstructive jaundice. In 1981 Pitt and colleagues (Pitt et al. 1981) defined eight factors predictive of mortality: the presence of malignancy, age greater than 60, albumin less than 3.0gm/dl, hematocrit less than 30%, white blood count greater than 10,000/mm^3, total bilirubin greater than 10mg/dl, serum alkaline phophatase greater than 100 IU, and creatinine greater than 1.3gm/dl. No mortality was encountered in patients with two or fewer factors. The presence of five factors was associated with 44% mortality, and patients with seven or eight factors all died. In 1987, Little estimated the risks of morbidity and mortality for patients undergoing surgery and percutaneous procedures for the relief of obstructive jaundice (Little 1987). The rate of bilirubin clearance (designated the k value) was 91% accurate in predicting outcome and a mortality index that used albumin, creatinine, and a cholangitis score was 100% accurate in assessing treatment risk. A recent retrospective study looking into the factors that predict early mortality following percutaneous endobiliary stent insertion in a small group of patients with jaundice found haemoglobin levels and blood urea to be independently significant in **predicting early mortality (ref.).** In addition, in this study the age and gender of the patient along with cancer type, level of obstruction, presence of pyrexia and bilirubin level had no influence on early mortality.

Tumour Factors

In addition to the patient-related factors, specific factors regarding each individual's tumour need to be considered before making a decision regarding operative exploration. The staging of patients with cholangiocarcinoma is usually accomplished using a combination of CT scan, MRI, cholangiography and angiography (Dooley et al. 1990).(de Groen et al. 1999b;Henson, Albores-Saavedra, & Corle 1992;Lynn, Wilson, & Cho 1988) Findings on CT scan such as bilobar peripheral hepatic metastases or extrahepatic disease preclude curative resection. Extensive bilobar hepatic parenchymal involvement also indicates unresectability. Atrophy of the lobe containing tumour with hypertrophy of the other side is also a sign that resection is not likely to be possible (Hadjis et al. 1989). The angiographic finding of tumour encasement or occlusion of the common hepatic artery or main portal vein is also considered by most groups as a contraindication to resection. Endoscopic ultrasound (EUS) has been reported to be of benefit in the staging of patients with extrahepatic cholangiocarcinoma (de Groen, Gores, LaRusso, Gunderson, & Nagorney 1999b;Tio et al. 1991;Wiersema et al. 1997). Laparoscopy as a staging procedure is practised in some centres to inspect the peritoneal cavity for the presence of metastatic deposits that fail to be detected by CT.

A 12-year study from Johns Hopkins compared the ability of cholangiography and angiography to determine resectability in 97 patients with pathologically proven cholangiocarcinoma. Overall, 62% of patients underwent complete (40%) or palliative (22%) resection, and 38% of patients underwent palliative procedures by operative (34%) or nonoperative (4%) techniques.

Cholangiographic involvement of four or more segments was observed in 19 patients (20%) and none of these were resectable. In comparison, resectability rates for patients with three or fewer (n=78), two or fewer (n=25), or only one segment (n=12) were 50%, 70% and 83% respectively. Overall, cholangiography predicted the clinical management in 60% of patients. Angiography demonstrated anatomic variations in 13% of patients, and findings of arterial or venous encasement in 33%. Angiography alone predicted the clinical management in 71% of patients, but the combined predictive value for cholangiography and angiography was 79%. These data suggest that both angiography and cholangiography should be performed preoperatively to stage patients with perihilar cholangiocarcinoma.

Surgical Resection

Intrahepatic Cholangiocarcinoma

Intrahepatic or peripheral cholangiocarcinoma is associated with a different clinical, laboratory and radiological presentation, and operative treatment also differs from hilar or distal duct cholangiocarcinoma. Intrahepatic cholangiocarcinoma is surgically approached in a fashion similar to hepatocellular carcinoma. In most cases hepatic resection is performed with standard resection techniques (Chen et al. 1989;Schlinkert et al. 1992). Care must be taken, however, to achieve a negative bile duct resection margin if the tumour approaches the hilum. When these tumours are resectable, negative margins can usually be obtained. The prognosis for resectable peripheral cholangiocarcinoma is more favourable than for hilar tumours (Klempnauer et al. 1997;Roayaie et al. 1998).

Hilar Cholangiocarcinoma

The biliary branches of the caudate lobe drain into the right and left hepatic ducts at the confluence. These ducts are often involved by hilar cholangiocarcinoma. Surgical resection of cholangiocarcinoma at this site without resection of the caudate lobe may leave a potential site for recurrent cancer. With tumour confined to the confluence of right and left hepatic ducts, caudate lobe and extrahepatic bile duct resection is required. However, when the main left and right ducts are involved by cholangiocarcinoma, caudate lobe resection should be performed along with resection of the involved liver parenchyma.

If the extension of the hilar cholangiocarcinoma is found predominantly in the left lateral and medial segmental ducts (segments ii. iii. iv), left hemihepatectomy along with caudate lobe resection should be performed. When the right anterior sectoral duct is also involved, extended left hepatic hepatectomy is recommended. On the contrary, if the cancer is predominantly in the right anterior and posterior segmental ducts, the right hepatic lobe and caudate lobe should be removed (Bengmark et al. 1988;Vauthey et al. 1993). Right and left percutaneous transhepatic catheters may be inserted preoperatively to assist with the technical aspects of resection (Crist, Kadir, & Cameron 1987;Yeo, Pitt, & Cameron 1990b).

The need for hepatic resection may be suspected at the time of preoperative cholangiography or angiography when neoplastic involvement of the right or left hepatic duct or portal vein is visualised radiographically. After resection of the hepatic duct bifurcation, biliary-enteric continuity is restored using a Roux-en-Y hepaticojejunostomy.

Another resection option for patients with hilar cholangiocarcinoma is total hepatectomy and liver transplantation. On the whole the results are disappointing, with many developing early and widespread recurrence (Goldstein et al. 1993) (Haug et al. 1992;Meyer, Penn, & James 2000;Ringe et al. 1989). Survival rates of less than 10% have been reported among all patients undergoing liver transplantation for cholangiocarcinoma. Despite these poor results, there are a number of reports showing favourable survival following transplantation in those group of patients undergoing liver transplantation for PSC (with incedental cholangiocarcinoma) or with early-stage disease (stages I and II) (Casavilla et al.

1997a;De, I et al. 2000;Iwatsuki et al. 1998).Overall, it is fair to say that liver transplantation stills plays a limited role in the treatment of cholangiocarcinoma.

Distal Cholangiocarcinoma

In patients with distal cholangiocarcinoma, pancreaticoduodenectomy serves as the optimal resectional therapy, with resection or preservation of the pylorus. The continuity of the gastrointestinal tract is most commonly performed using pancreaticojejunostomy, hepatoicojejunostomy, and duodenojejunostomy.

Diagnostic methods and the ability to perform curative surgical procedures for biliary duct malignancies have improved in recent years. However, the difficult problems of resecting hilar cholangiocarcinoma with clear margins have remained. Detailed preoperative evaluation with PTBD, PTCS, US, CT and angiography are performed by some groups allowing an accurate diagnosis of the proximal extent of hilar cholangiocarcinoma along the intrahepatic segmental bile ducts and direct invasion of surrounding organs. Such preoperative information allows the design of an operative procedure for each patient before surgery (Nimura et al. 1991a). In well selected cases radical resection that include resection of portal vein and/or head of pancreas with negative hilar nodes have produced some cases of long term survival following resection of cholangiocarcinoma (Nimura et al. 1991b) (Nimura et al. 1998).

Palliative Therapy

Nonoperative palliation

Palliative therapy in patients with cholangiocarcinoma includes both operative and nonoperative procedures. The aim of either approach is to relieve the most troublesome symptom, which is pruritus secondary to biliary obstruction. A nonoperative approach for sympton control may be undertaken because the patient is unfit for surgery or because the tumour is unresectable by staging investigations. Criteria that exclude patients from surgery include poor general medical condition, refusal of surgery, distant metastases, tumour extension into the secondary biliary radicals of both the left and right lobes of the liver, and portal vein or main hepatic artery encasement. Patients undergoing nonoperative palliation may have their biliary decompression performed using either percuteaneously placed or endoscopically placed drainage catheters (Coons 1992;Kubota et al. 1993;Lai et al. 1992;Nordback et al. 1994;Tio, Cheng, Wijers, Sars, & Tytgat 1991). Percutaneous catheters can be used for lesions at any site. The endoscopic approach on the other hand may be best reserved for distal inoperable cholangiocarcinoma.

Biliary Drainage

In the last 15 years, advances in percutaneous technique and imaging have enabled the interventional radiologists to become an integral part in the management of biliary malignancies. In hilar cholangiocarcinoma, the percutaneous approach is useful in the diagnostic workup, defining the biliary anatomy prior to surgery, providing temporary or longterm biliary drainage, and providing access for percutaneous radiation therapy.

Percutaneous techniques

Percutaneous transhepatic cholangiography (PTC) and percutaneous biliary drainage (PBD) are used in the diagnosis and treatment of malignant biliary obstruction. Prior to performing these procedures, patients should have any clotting abnormalities corrected and be on antibiotics. Up to a third of patients known to have malignant biliary obstruction have infected bile. Thus, broad spectrum, parenteral antibiotics should be administered pre-procedure ideally for 24hrs. The procedure can be carried out under local anaesthetic.

Traditionally, biliary drainage has been performed under fluoroscopic guidance. More recently, ultrasound guidance is used for initial insertion of a 22-gauge needle into the left and/or right biliary radicals. This is then followed by cholangiography and fluoroscopy.

Percutaneous biliary drainage may be performed either as a temporary or a long term procedure in patients with malignant biliary tract obstruction. Temporary drainage is usually chosen in patients in whom further therapeutic manoeuvres such as surgery, chemotherapy, or radiotherapy are being considered. Long term biliary drainage is performed as a palliative procedure in patients with advanced cancer.

Temporary Biliary Drainage

The notion that preoperative biliary drainage reduces the morbidity and mortality of subsequent palliative or curative surgery for biliary obstruction has been seriously questioned. Risk factors in patients undergoing surgical decompression of the biliary tree include sepsis and renal failure. Although PBD lowers the serum bilirubin level and improves both renal and hepatic function, there is no evidence that this

translates into better prognosis for those patients that later undergo surgery. In addition, the risks of PBD must be taken into account when assessing its potential benefits preoperatively.

Placement of a PBD catheter may also be useful during surgery in patients undergoing complex biloenteric anastomoses. The surgeon can palpate the catheter at surgery thereby assisting in identifying structures. However, some surgeons consider that preoperative biliary drainage increases the difficulty of surgery by reducing the size of the bile ducts making biloenteric anastomoses more difficult and introducing infection into the biliary tree. Patients with malignant biliary obstruction secondary to tumours that are particularly responsive to chemotherapy or radiotherapy such as lymphoma or oat cell metastases are potential candidates for temporary PBD. The procedure may be indicated because of pruritis and/or cholangitis prior to definitive therapy. In addition, patients who require chemotherapeutic agents that are excreted into the bile also may benefit from temporary PBD.

Longterm percutaneous biliary drainage

The indication for PBD in the longterm palliative treatment of malignant biliary obstruction must be considered with respect to available surgical and endoscopic alternatives. The choice of PBD versus endoscopic drainage is largely dependent on availability of local expertise. However, successful endoscopic stenting is more likely with a distal rather than proximal biliary stricture.

The prognosis for cholangiocarcinoma at the hilum is not as favourable as in the distal duct. Extension of the tumour proximally leading to bilateral involvement of the

hepatic ducts beyond the second order branches may preclude curative resection. Cholangiography typically underestimates the extent of disease. In patients who are not surgical candidates, complete drainage of both left and right lobes can be achieved with PBD using one or multiple catheters. Using endoscopy, drainage of the right and left tree is accomplished only in 25% of patients. An undrained or partially drained system leaves the patient at risk of developing cholangitis.

When obstruction to mutiple segmental ducts exists, life expectancy may be limited and percutaneous drainage with multiple catheters may not improve the length or quality of life and the risk of cholangitis is outweighed by intervention. In such patients, limiting the number of catheters inserted is the preferred option. Percutaneously placed catheters may also be used as conduits for the application of local radiotherapy, such as Iridium-192 wires (Karani et al. 1985;Milella et al. 1998).

Complications after PBD may be classified as acute or delayed. Acute complications occur in 5-20% of patients and include bleeding, septicaemia, bile peritonitis and procedure related death (Mueller et al. 1985). Preventive measures to avoid haemorrhage include correction of clotting abnormalities, the use of small calibre coaxial systems, and puncturing a peripheral duct to prevent damage to a central large vessel. Sepsis is avoided by the use of prophylactic antibiotics, avoidance of over distension of the biliary tree during PTC and PBD and ensuring minimal manipulation in an infected system. Avoiding an extrahepatic puncture and performing a PBD if the PTC shows a high level of obstruction is thought to reduce the incidence of biliary peritonitis. Likewise, correct positioning of the catheter side holes such that none are extrahepatic prevents bile peritonitis.

Delayed complications of PBD include cholangitis secondary to catheter occlusion (4-45%), catheter dislodgement (10-20%), and pericatheter leakage (10-30%) (Mueller, Dawson, et al 1985). Regular irrigation with 3-5mls of sterile saline, the use of larger calibre catheters (10-14 F), elective catheter exchanges every 2-4 months and preprocedural antibiotics during catheter exchange help to reduce the incidence of delayed complications. In addition, locking catheters effectively prevents the dislodgement. Local skin infection, granulomas, and tumour tracking at the catheter entry site occasionally may occur.

Biliary endoprostheses

Percutaneous biliary drainage requires a protruding catheter with associated problems that include local pain, irritation, infection, peritubal leakage and catheter dislodgement. To overcome these problems an indwelling stent (endoprosthesis) was proposed as an alternative to catheter drainage (Burcharth 1978;Pereiras, Jr. et al. 1978). The ideal stent should bypass the obstruction, remain in place, and maintain patency.

After initial enthusiasm for stent placement, it soon became clear that these devices were far from ideal (Mendez, Jr. et al. 1984). More recently, expandable metal stents have been introduced as an alternative to conventional plastic stents to reduce problems with longterm patency and stent migration.

The decision to place an indwelling biliary endoprosthesis requires an individually tailored approach. The mean duration of patency for a plastic endoprosthesis is 5-6 months (Lammer & Neumayer 1986;Lammer, Neumayer, & Steiner 1986). Therefore a patient with a short life expectancy is more suitable for this technique.

Expandable metallic stents are currently being used increasingly in the treatment of malignant obstruction. The rationale for their use is the ability to introduce a large diameter stent using a small introducer system. Stents are compressed prior to insertion and expand to their full diameter only after deployment.

The expandable luminal diameter of metallic stents is 10-12mm. This compares favourably with the diameter of conventional plastic stents (4-7mm). In view of the recognised correlation between luminal diameter and patency rates, the larger diameter metallic stents should improve patency. Reported rates of occlusion vary from 6-50% (Irving et al. 1989;Lameris et al. 1991). The mean patency rate has been reported to be 4-7 months.

The advantages to metal stents are that they can be introduced via a 7-10 F tract rather than the 14-16 F tract required for plastic stents. This offers several potential advantages including a reduction in pain and discomfort during tract dilatation, a decrease in the number of early complications, and the ability to perform biliary drainage and stent insertion as one procedure. In contrast to plastic stents, migration has not been a problem with metal stents. The disadvantage to metal stents is they are more expensive than plastic stents and once inserted cannot be removed. Confirmation of diagnosis should therefore always be obtained before metal stent deployment.

The treatment of hilar cholangiocarcinoma is challenging. Percutaneuos intervention techniques such as PTC, PBD and insertion of stents are techniques available to the interventional radiologist. A team approach between surgeons, endoscopists, and interventional radiologists will often lead to the best results.

Operative palliation.

In good risk patients without preoperative evidence of metastatic or locally unresectable disease, surgical exploration is undertaken (Yeo, Pitt, & Cameron 1990b). The use of laparoscopy as a staging tool reduces the number of unnecessary laparotomies. In those cases with no evidence at laparoscopy of peritoneal spread surgical exploration is performed. There will be some cases with no evidence of advanced disease on laparoscopy that are found at the time of surgery to have peritoneal seedlings hidden from the view of the laparoscope or cases where advanced local disease precludes resection (Weber et al. 2002). In such cases any stents placed preoperatively are left in place while the gallbladder may be removed in order to prevent the subsequent development of acute cholecystitis from cystic duct obstruction related to stenting (Lillemoe et al. 1993a). Postoperatively stents can be exchanged under fluoroscopic guidance for larger diameter ones.

Most centres as a matter of routine now perform staging laparoscopy to identify those patients with peritoneal involvement.

In patients with locally advanced unresectable tumours, several noncurative approaches are available for palliation. Options for perihilar tumours include: (1) Roux-en-Y hepaticojejunostomy with intraoperative placement of silastic transhepatic stents, and (2) segment III bypass to the left intrahepatic ducts. For distal tumours, choledochojejunostomy and gastrojejunostomy are usually the procedures of choice.

Although symptomatic gastroduodenal obstruction is uncommon at the initial diagnosis of cholangiocarcinoma, some reports suggest up to a third of

patients with unresectable tumours develop bowel obstruction before death (Guglielmi et al. 1997). For this reason, gastrojejunostomy should be considered. Another major problem for patients with unresectable lower common bile duct tumours can be epigastric and back pain. Chemical sympathectomy using 50% alcohol can be performed intraoperatively to achieve pain control (Lillemoe et al. 1993b;Yeo, Pitt, & Cameron 1990b).

Data comparing the morbidity and mortality rates between operative and nonoperative palliation in cholangiocarcinoma is lacking due to the lack of clinical trials (Nordback et al. 1994).

Palliative Therapy

Chemotherapy

The use of chemotherapy alone, using 5 Fu and multiple other drugs, has not been shown to improve survival in patients with either resected or unresected cholangiocarcinoma (Poplin et al. 1999a;Ravry et al. 1986;Sanz-Altamira et al. 1998a). Most of the chemotherapy trials performed in patients with biliary tract malignancies have included patients with both gallbladder or/and bile duct cancers with response rates at best between 10 to 20%. Recent trails using some of the latest chemotherapy agents such as paclitaxel and gemcitabine have also shown disappointing results (Bukowski, Leichman, & Rivkin 1983a;Pazdur et al. 1999a;Sanz-Altamira et al. 1998b). There is a need for studies into new strategies aimed at improving responses to chemotherapy.

Radiotherapy

Radiation therapy has been evaluated in patients with cholangiocarcinoma: external beam radiotherapy, intraoperative radiotherapy, and internal radiotherapy, radioimmunotherapy and charged particle radiation. External beam radiotherapy has most frequently been delivered through a multishaped portal using three or four fields with standard fractions (1.8 Gy/day) to a total dose of 45-60 Gy (Hayes, Jr., Sapozink, & Miller 1988;Shiina et al. 1992;Verbeek et al. 1991). Custom blocking is used to limit the dose to the right kidney, duodenum, liver, small bowel and the spinal cord. Intraoperative radiotherapy (Busse et al. 1989;Ede et al. 1989;Monson et al. 1992) has typically been administered to a total dose of 5 to 20 Gy and is generally followed by additional external beam. Internal radiotherapy

(Cameron et al. 1990a;Koyama et al. 1989) is normally delivered through either percutaneous or endoscopically placed biliary stents, using iridium 192 or cobalt 60 as the radiation source. Total radiation doses may vary from 20 to 60 Gy at 0.5 to 1.0 cm from the source. Some groups (Minsky et al. 1991;Robertson et al. 1993) have used combined radiotherapy and chemotherapy but no controlled data are available. Radioimmunotherapy (Stillwagon et al. 1991) has been used at Johns Hopkins for the treatment of unresectable intrahepatic cholangiocarcinoma using iodine 131 anti-CEA as a component of the therapy. In addition, charged particle radiation using helium or neon ions has been used at the Lawrence Berkeley Laboratories in patients with perihilar tumours (Schoenthaler et al. 1994).

To date, no prospective, randomised trials of the use of radiotherapy have been reported. One of the largest retrospective studies (Cameron et al. 1990b) analysed 38 patients that had resection of their perihilar tumours along with external beam radiotherapy. 29 of these patients also had iridium 192 seeds delivered to the tumour bed through transhepatic stents. When compared with 15 resected, but not irradiated, the median survival was identical, but the only 5-year survivors were in the irradiated group. However, 10% of the radiated group had late problems with duodenal obstruction or bleeding. Similarly, 25 unresected patients receiving external beam radiation were compared with 18 unresected patients who did not receive radiotherapy. In this analysis, survival was significantly improved ($p < 0.05$) among the unresected patients who received radiotherapy. As with all retrospective analysis, however, these data are suspect because the groups, even stratified by resection, were not comparable. In general,

patients chosen to receive radiotherapy were healthier and had more localised or smaller tumours. A more recent 5-year analysis from the same centre (Pitt et al. 1995) focused on 50 operatively staged patients with perihilar cholangiocarcinoma who had no evidence of metastatic disease, and a Kanofsky score greater than 60. 23 of these patients, who received between 45 to 55 Gy postoperatively, were comparable by stage of disease, type of surgery, and multiple other parameters to 27 patients who did not receive radiotherapy. In this analysis, no survival advantage was achieved in the patients treated with radiotherapy. These data suggest that radiotherapy alone is not helpful as an adjuvant therapy and may need to be given in combination with other treatments or as neoadjunct therapy.

Photodynamic therapy (PDT)

PDT is a novel form of cancer treatment that has been found to offer good palliation of cholestasis, improve quality of life, and possibly increase survival in patients with advanced and unresectable cholangiocarcinoma (Hochberger & Hahn 1992;Ortner et al. 1998). This form of therapy relies on the cancer cells taking up photo-activated compound such as photofrin, which is then activated by irradiation resulting in the killing of the cancer cell (Pahernik et al. 1998;van den Boogert et al. 1998). Although there are only a few published reports of the benefit of this form of therapy in cholangiocarcinoma, this is an interesting new approach in the palliative treatment of cholangiocarcinoma.

1.2 APOPTOSIS

1.2.1 History

Until recently, cancer was thought of, in almost exclusive terms, as a disease of cell proliferation. However, developments within the cell death field over the past decade has provided a new perspective into how cell populations are normally maintained at equilibrium and have revealed how defects in cell death regulation (apoptosis) can contribute to the development of malignancy.

The distinct morphology of the process now known as apoptosis had been recognised as early as the turn of the century. In 1885, Walther Flemming, who introduced the term chromatin and mitosis, published a drawing of apoptosis occurring during the regression of ovarian follicles. He called it chromatolysis. In 1914, a German anatomist, Ludwig Grapper, pointed out that a mechanism must exist to counterbalance the mitosis and proposed Flemming's chromatolysis as being the answer (Majno & Joris 1995). The general significance of these reports was unrecognised by the majority of scientists. In 1965, Kerr et al introduced the term shrinkage necrosis to describe the cell death he observed in rat liver following portal vein occlusion. This form of cell death differed from classical necrosis in histological appearance, affecting only scattered single cells and not being associated with inflammation (Kerr 1965). "Shrinkage necrosis" was soon found to occur in sites other than the liver such as in basal cell carcinoma (Kerr & Searle 1972). Further studies by Kerr and colleagues during the mid 1960s and early 1970s culminated in what has become a landmark paper (Kerr & Searle 1972) in which the term apoptosis was proposed for those forms of cell death that shared a distinctive set of morphological characteristics. It was only around the mid 1980s that other scientists began to take interest in apoptosis. In the mean

time, Wyllie, Kerr and Currie continued to work together adding to their initial observations. It was largely immunologists, intent on understanding cytotoxic lymphocyte-mediated killing and thymic selection, who were the ones to finally appreciate the wider significance of the work by Kerr et al that helped bring this topic to the wider scientific community

Apoptosis is fundamental to processes as diverse as tissue remodelling during embryogenesis, maintenance of tissue homeostasis in the adult and to both innate and cognate immunity. A cell upon receipt of an appropriate stimulus, engages a set of molecules that cooperate to dismantle the cells from within. The process culminates in a cell exhibiting characteristic changes in appearance, notably plasma blebbing, chromatin condensation and formation of apoptotic bodies (Kerr, Wyllie, & Currie 1972;Wyllie, Kerr, & Currie 1980). The alteration to the composition of the plasma membrane of the cell that occurs in the later stages of apoptosis attracts the attention of nearby phagocytes. These phagocytes then eat the dying cell- and this is a critical point – before it has a chance to burst and release its contents into the extracellular space. Thus, apoptosis can be viewed as a kind of packaging process that facilitates the disposal of unwanted cells by breaking them up into "bite-sized" pieces, complete with the attached label which reads "I'm dying, come and get me!" The membrane changes that trigger macrophages and other cells with phagocytic capability to eat apoptotic cells remain incompletely understood; however, two main changes have been consistently observed. Savill, Haslett and colleagues have identified a mechanism that utilises thrombospondin, in concert with CD36 and the vitronectin receptor, to recognise a thrombospondin-binding moiety that appears on apoptotic cells from

many lineages (Savill et al. 1990). Another subset of phaghocytes appear to recognise apoptotic cells due to the appearances of phosphatidylserine – a lipid normally confined to the inner leaflet of the plasma membrane – on the external leaflet of the plasma membrane of these cells (Fadok et al. 1992;Martin et al. 1996a;Savill, Dransfield, Hogg, & Haslett 1990).

Part of the cellular dismantling process involves the destruction of the normal nuclear architecture and cleavage of chromatin (Savill, Dransfield, Hogg, & Haslett 1990;Wyllie, Kerr, & Currie 1980). It is these changes that result in the striking phenotypic alteration that were originally recognized by Kerr and colleagues (Kerr, Wyllie, & Currie 1972) to be characteristic of apoptotic cells. Collapse of the nucleus is thought to be due to destabilisation of the nuclear envelope as a result of lamin proteolysis. This results in loss of matrix attachment regions – points at which the chromatin is attached to the nuclear envelope – causing the chromatin to compact (Neamati et al. 1995). A wide range of physiological stimuli or experimentally applied stress conditions can induce apoptosis, and this appears to be conserved through evolution. Apoptosis can be induced via multiple independent pathways with distinct signalling intermediates. These various routes all converge upon activation of caspases, leading to breakdown of vital cellular substrates resulting in the demise of the cell (Jacobson 1997).

Apoptosis is currently one of the fasted growing fields in biology with spectacular progress made over the last few years in identifying and understanding the molecular mechanisms that underlie this process. It is now indisputable that

apoptosis plays an essential role in normal cell physiology and that aberrant apoptosis can manifest itself in a variety of human disorders.

Apoptosis and Necrosis

Broadly speaking, apoptosis is the mode of cell death that is observed where death is a normal part of the lifecycle of the organism and for this reason has been called "the physiological mode of cell death." This is not strictly true, since apoptosis is frequently observed when cells are dying due a pathological process – during viral infection for example (Terai et al. 1991). Because apoptotic cells are eaten before they can release their contents into the extracellular space, damage to neighbouring cells is largely curtailed – this is a critical difference between apoptosis and necrosis (Kerr, Wyllie, & Currie 1972;Searle, Kerr, & Bishop 1982).

Necrotic cell death is generally the result of a gross departure from physiological conditions where the cell suffers a major insult (Lennon, Martin, & Cotter 1991). Damage is generally so severe that the cell loses its ability to maintain membrane integrity within a matter of minutes, with the result that it undergoes rapid swelling (due to the influx of water) and bursts open, thus releasing its contents. Cells contain many enzymes (lipases, proteases, nucleases) and byproducts of metabolism (such as reactive oxygen species) that can cause severe cellular injury and even cell death if these are simply released into the extracellular space. Thus, a necrotic cell often ends up damaging many neighboring cells as a consequence of its demise. This sets up a chain reaction cell death, as the wave of necrosis radiates out from the initial site of damage. To make matters worse, all of the dead and dying cells soon attract the attention of neutrophils and inflammatory macrophages that rush in expecting to find a bacterial infection or some other invasion. Confronted by this unsightly mess they

release their own toxic enzymes, thereby adding to the carnage. This scenario reveals why necrosis was for a long time considered tobe the predominant mode of cell death – it was difficult to miss! By contrast, apoptosis can occur at high frequency within tissues and go practically unnoticed due to the efficiency of clearance of the dead cell corpses. In summary, having a more controlled mode of cell death that prevents the escape of cellular contents, the body avoids not only the injury and death of cells surrounding the cell destined to die, but also the further cell death and wasted effort that results from mounting an inflammatory reaction.

1.2.2 Molecular Mechanisms of Apoptosis

Apoptosis has a central role in the normal development and homeostasis of all multicellular organisms. It is also used by the body's defence system to eliminate dangerous cells, such as those that are either mutated or are harbouring viruses. Deregulation of this process, resulting in either too much or too little cell death, can cause both development defects and a wide variety of disease states. Much of the understanding of apoptosis has come from genetic studies in the nematode *Caenorhabditis elegans*. Although the mechanisms of apoptosis are highly conserved, regulation of apoptosis in man is more complicated and involves several large families of proteins.

Although the first cell death gene to be identified, Bcl-2, was a mammalian gene, much of the genetic framework of the process of apoptosis was learnt from the study of the nematode *C. elegans* (Ellis & Horvitz 1986) (Hengartner, Ellis, & Horvitz 1992), in which cell death mutants can easily be generated. Studies show

that during development of the *C. elegans* hermaphrodite, exactly 131 of the 1090 somatic cells formed undergo apoptosis. Two genes, *CED-3* and *CED-4* are essential for all developmental programmed cell death in *C. elelgans.* Cells destined to die, survive in worms with loss of function of either of these genes (Ellis & Horvitz 1986). CED-3 encodes a cysteine protease or caspase for which there are multiple human homologues (Yuan et al. 1993). The caspases are key death effector molecules and their activation, which can be induced in multiple ways, is required for cell death.

The CED-4 protein appears to function as an adaptor protein that activates the CED-3 caspase precursor. These two proteins bind to each other through a shared caspase recruitment domain (CARD) in the n-terminus of CED-4 and the pro-domain of CED-3 (Hofmann, Bucher, & Tschopp 1997;Irmler et al. 1997).

The product of the gene CED-9 negatively regulates apoptosis. (Hengartner, Ellis, & Horvitz 1992;Shaham & Horvitz 1996). Genetically CED-9 acts upstream of CED-4 and CED-3, as loss of function mutations in the CED-9 gene result in excessive cell death during development and embryonic lethality, and this excessive cell death can be suppressed by mutations of either CED-3 or CED-4. Conversely, a gain of function mutation in the CED-9 prevents cell death during development.

As CED-9 can bind to CED-4, its protective properties are most likely due to inhibition of ced-4 activity, preventing it from activating the caspase ced-3 (Chinnaiyan et al. 1997;Wu, Wallen, & Nunez 1997). Ced-9 may act by sequestering ced-4 from the cytosol and relocating it to the intracellular membranes (Wu, Wallen, & Nunez 1997). Ced-9 does not appear to function by

competition with ced-3 for ced-4 binding since simultaneous interaction of both molecules with ced-4 has been demonstrated (Chinnaiyan, O'Rourke, Lane, & Dixit 1997).

There are nine known mammalian homologues of ced-9. As one of these, Bcl-2, can inhibit programmed cell death in c. elegans (Vaux, Weissman, & Kim 1992) and can rescue a ced-9 mutant worm (Hengartner & Horvitz 1994a). It is likely that the mammalian Bcl-2 family proteins act just like ced-9, namely to inhibit apoptosis by preventing caspase activation caused by mammalian adaptor proteins that function analogously to ced-4. To date, one mammalian homologue for ced-4 gene (Apaf-1) has been described which seems to be required for the activation of caspase 3 in a cytochrome c dependent manner (Zou et al. 1997).

Genetic studies in C. elegans have also shown that multiple genes are needed for the efficient engulfment of the dead cell corpse and degradation of the dead cell's DNA. These genes, which presumably encode "eat-me" signals on the dead cell and receptors on the engulfing cell, allow efficient recognition and removal of apoptotic bodies. One gene, *nuc-1,* encodes a nuclease that may function analogously to the endonuclease that causes DNA degradation ("Laddering") in apoptotic mammalian cells (Hedgecock, Sulston, & Thomson 1983).

The caspases

As in C. elegans, the key effector proteins of apoptosis in mammalian cells appear to be caspases, cysteine proteases that have an aspartate specificity.

The first to be identified was the IL-1β converting enzyme (ICE), which, as the name suggests, was not identified by virtue of its death-promoting activity,

but by its ability to cleave the inactive precursor of IL-1β into an active cytokine (Cerretti et al. 1992). Indeed, the apoptotic properties of ICE (now called caspase-1) were not recognised until after the characterisation of the C. elegans gene ced-3, which bore striking homology to ICE (Yuan, Shaham, Ledoux, Ellis, & Horvitz 1993).

Ten mammalian caspases have been identified to date (Henkart 1996). All caspases cleave after aspartate residues but differ in their specificity for preceding amino acids as well as the overall tertiary structure of the substrate polypeptide. For example, while caspase-1 preferentially cleaves after YVAD sequences, another mammalian caspase CPP32 or caspase-3, similarly to ced-3 protein, cleaves preferentially before YVAD.

All caspases are produced as inactive precursor proteins that must be processed by proteolytic activity into the active enzyme. The processing of caspases is best defined for caspase-1 (Walker et al. 1994). From the 45-kDa caspase-1 precursor, the pro-domain is removed and the remaining protein processed into a p-10 and p-20 subunit. Two of each of these subunits is assembled into the active heterotetramer. Four cleavage events are required to yield active ICE, and each occurs after an aspartate residue.

The fact that caspase-1 is required for generating the pro-inflammatory cytokine IL-1β illustrates that, contrary to earlier beliefs, apoptosis does not always occur quietly and in the absence of inflammation (Cerretti, Kozlosky, Mosley, Nelson, Van Ness, Greenstreet, March, Kronheim, Druck, Cannizzaro, & et al. 1992). It is possible that some caspases will be used to cause apoptosis in normal circumstances such as development, when inflammation is not desirable.

On the other hand, others, such as ICE, may be used in cases such as defence against viruses, when inflammation and involvement of the immune system is helpful.

Gene deletion (knockout) experiments in mice have shown that mice lacking the gene for caspase-1 develop normally but cannot produce active IL-1β (Kuida et al. 1995). Mice lacking the gene for caspase-3 are born with giant brains, due to failure of excess neuronal cells to die normally during development (Kuida et al. 1996). It is likely that caspase are not the only important proteases in apoptosis. Studies with protease inhibitors have implicated other proteases including other cysteine proteases, aspartic proteases, serine proteases and the proteosome (Deiss et al. 1996;Martin et al. 1996b;Shi et al. 1992). The caspases are key death effector molecules and their activation, which can be induced in a multitude of ways, is required for cell death.

Caspase Substrates (Executors of Apoptosis)

Gene deletion studies in C. elegans and in mice have shown that certain caspases are required for apoptosis. These caspases must cleave polypeptide substrates within the cell to cause the morphological changes recognised as apoptosis. The nature of these substrates is an area of intense investigation.

Some caspases are known to be able to cleave and thereby activate the precursor of other caspases. For example, caspase-2, -6, -8 and -10 can cleave and activate pro-caspase-3 (Fernandes-Alnemri et al. 1996;Liu et al. 1997;Muzio, Salvesen, & Dixit 1997). Some active caspases can activate their own precursors, for example caspase-1

can cleave pro-caspase-1. In this way it is possible that the caspases act in a hierarchical fashion resulting in an avalanche of protease activity.

A number of other substrates of the caspases have been identified that are cleaved during apoptosis. So far, the only one that is thought to have its main role in cell death is DNA fragmentation factor (DFF) (Liu, Zou, Slaughter, & Wang 1997). DFF is a heterodimer that is activated following its cleavage by caspases, and is required for subsequent activation of the endonuclease that causes the DNA fragmentation that usually accompanies apoptosis.

The growing list of other proteins that can be cleaved by caspases include, poly-ADP-ribose polymerase (PARP), DNA protein kinase, lamins A and B, protein kinases A and C, actin and retinoblastoma protein (Emoto et al. 1995;Goldberg et al. 1996;Lazebnik et al. 1994). There is currently controversy over whether one or a few of these substrates must be cleaved for apoptosis to occur, or whether many proteins are cleaved during apoptosis but none has special significance.

Even when it is known that these proteins play no essential role in apoptosis, cleavage of particular proteins can be used as a marker of caspase activation and apoptosis. For example, PARP is not essential for apoptosis as PARP knockout (KO) mice are normal, but detection of cleaved PARP has been used as an indicator that caspases such as CPP32 have been activated (Fernandes-Alnemri et al. 1995).

Adaptor Proteins: The caspase Activators

Although the mammalian homologue of ced-4, apaf-1, has not yet been shown to act as an adaptor molecule, a number of adaptor molecules have been found that function as caspase activators. Like ced-4, these proteins are thought to bind to the

pro-domain of the caspase precursors through homotypic interaction motifs, ultimately leading to cleavage and activation of the caspase.

The first mammalian cell death adaptor molecule to be identified was MORT-1/FADD (Boldin et al. 1995;Chinnaiyan et al. 1995). FADD possesses a motif known as the death effector domain (DED), which interacts with DEDs in the pro-domain of caspase-8 (Boldin et al. 1996). Subsequent proteolytic processing either by autoprocessing or by another protease releases the active form of caspase-8 from the receptor complex (Medema et al. 1997). The other end of FADD bears another interaction motif termed a "death domain" (DD) that allows it to bind to signalling molecules and receptors sending the death signal. FADD therefore acts as an adaptor molecule by coupling the death signalling molecules to the caspases via its DD and DED domains.

Another adaptor molecule is RAIDD, which, like FADD, has a death domain (DD) at one end (Duan & Dixit 1997;Hofmann, Bucher, & Tschopp 1997). The other end of RAIDD does not bear a DED domain, but has a "caspase recruitment domain" (CARD). The CARD motif in RAIDD allows it to bind the CARD motif in the pro-domain of caspase-2 (Nedd-2) in a manner analogous to the binding of the DED of FADD to the DED domains in caspase-8. CARD domains have also been identified in the pro-domains of ICE and *ced*-3.

1.2.3 Extracellular Regulators of Apoptosis (Receptor-mediated Signal Transduction)

The signal for cell death can be generated in a vast number of ways. In normal circumstances it is often generated by the addition or removal of cytokines such as tumour necrosis factor (TNF) or growth factors. Death signalling pathways activated by members of the TNF receptor family, which include CD95 (Fas/Apo-1), are understood in the greatest detail. The cytoplasmic domain of CD95 possesses a death domain (DD). Ligand binding induces the DD in the cytoplasmic domain of CD95 to associate with the DD in FADD (Kischkel et al. 1995). Ligand binding is thus able to stimulate the formation of a death signalling complex that recruits both FADD and caspase-8, which then become activated (Boldin, Goncharov, Goltsev, & Wallach 1996;Muzio, Salvesen, & Dixit 1997). Many cells are dependent on growth factors, and undergo apoptosis when they are removed. The molecular mechanism involved in the regulation of cell survival by growth factors is not known in any detail, but it is known that apoptosis occurs through caspases, and in most cases death can be inhibited or blocked by Bcl-2.

Some death signals originate within the cell. Disturbances to the cell cycle, such as caused by overexpression of c-myc in resting cells, or radiation/chemotherapy induced DNA damage, can induce apoptosis through p53 dependent and independent pathways. This pathway is inhibited by Bcl-2 (Ryan et al. 1994). Viruses can elicit a defence apoptotic response in infected cells as an altruistic attempt to stop virus spread (Lowin et al. 1994). Presumably the cell detects changes to cellular metabolism caused by the virus and then kills itself. Thus a cell may kill itself as a response to stress, and this may explain the

propensity of cells to undergo apoptosis when exposed to a variety of drugs and toxins with widely varying pharmacological actions (Vaux & Hacker 1995). The fact that some inhibitors of apoptosis are able to block apoptosis irrespective of the initiating stimulus suggests the presence of a common pathway. Recent evidence supports a role for the mitochondria as central players in apoptosis. Studies on isolated mitochondria show that release of the caspase activator cytochrome c from the intermembrane space of this organelle, is a critical step in the induction of apoptosis (Green & Reed 1998a;Kantrow & Piantadosi 1997;Kroemer, Dallaporta, & Resche Rigon 1998;Martinou 1999).

1.2.4 Intracellular Regulators of Apoptosis (Bcl-2 Protein Family and p53 protein)

The Bcl-2 family is a growing group of proteins (Boise et al. 1995;Wang & Reed 1998). These proteins are critical regulators of the programmed cell death pathway. The expression of these proteins frequently becomes altered in human cancers, thus contributing to neoplastic cell expansion by prolonging cell survival. Bcl-2, a 52 KDa protein, the prototype of this family was first discovered when several groups cloned the t(14;18) chromosomal translocation commonly found in human follicular B cell lymphoma (Reed et al. 1989). This translocation was found to dysregulate the expression of Bcl-2 by placing the coding region of Bcl-2 under the control of the immunoglobulin heavy chain enhancer (Eμ). This translocation usually results in aberrant overexpression of Bcl-2 (Graninger et al. 1987;Tsujimoto 1989). Since then however, overexpression of Bcl-2 has been reported in a variety of cancers, including prostate, colorectal, lung, renal and other types of solid tumours and leukaemias. Experiments in which Bcl-2 was

overexpressed in growth-factor-dependent cell lines gave the first clues to its function. Later studies including cells of transgenic mice showed that Bcl-2 had the ability to protect cells from apoptosis induced by a wide range of stimuli (Sentman et al. 1991). Cloning of the Bcl-2 gene opened up the field of apoptosis genetics and its role in the field of cancer. A variety of experiments have provided conclusive evidence that the elevations in the expression of Bcl-2 cause resistance to chemotherapeutic drugs and radiation, while decreases in Bcl-2 expression promote apoptotic responses to anticancer drugs and radiotherapy.

Structure

A number of genes similar to Bcl-2 have been discovered in mammals, some with extensive sequence homology and others more distantly related (Reed 1997a;Yang & Korsmeyer 1996). Detailed characterisation of the amino acid sequences of Bcl-2 family members, their biochemical action and their function has led to three key observations:

1. There are conserved regions in Bcl-2 family members, four BH (for Bcl-2 Homology) domains, and the membrane-spanning domain (TM).
2. There are multiple types of physical interaction between proteins of the Bcl-2 family.
3. There are anti-apoptotic (Bcl-2, Bcl-xL, Mcl-1, survivin, Bcl-w, A1, Ced-9) and pro-apoptotic members (Bax, Bad, Bak, Bid, Bik/Nbk, Bok)

An important observation drawn from the discovery of new Bcl-2 family members was not only that they contained conserved regions, but also that not all of those

regions were present in every family member. This meant that differences in function could be quickly assigned to conserved regions. Without this information, painstaking random mutagenesis would have been necessary for structure/function studies, since the sequences of Bcl-2 family members have none of the characteristic motifs that provide evidence for some enzymatic activity or indicate relatedness and thus similar function to other known proteins. Four conserved domains have so far been identified in Bcl-2 family members. These regions have been denoted (from the N-terminus) BH4, BH3, BH2 and BH1. The numbering reflects the order of discovery of these domains. The anti-apoptotic members of the Bcl-2 family characteristically share at least three, and usually four, of the BH regions, which is reflected in the significant overall similarity between these proteins. The fact that A1 and several viral Bcl-2 homologues function as anti-apoptotic proteins (Lin et al. 1996;Nava et al. 1997) without a recognised BH3 region indicates that this region is not absolutely required for survival function. On the contrary, little overall sequence similarity is required for similar function among the pro-apoptotic Bcl-2 family members. Since all the pro-apoptotic proteins so far discovered have a BH3 region, and not all of the anti-apoptotic proteins have this region, this may be a defining characteristic for the two sub-families. The limited common features of the pro-apoptotic members of the Bcl-2 family (all they share is a 16 amino acid BH3 domain) may mean that each has some specific activity, such as interaction with specific upstream signalling molecules, and their common function is to bind and antagonise the anti-apoptotic effects of their ligands. This implies that the biochemical effects of the Bcl-2 family on apoptosis are mediated through the anti-apoptotic members. This has

important implications when suggesting a model of how the Bcl-2 family may regulate apoptosis.

Many Bcl-2 family members have a conserved carboxyl-terminal transmembrane region. This region localises proteins to the outer leaflet of the endoplasmic reticulum, nuclear and mitochondrial membranes (Hockenbery et al. 1990;Krajewski et al. 1993a). Such a location is a likely site for intrafamily interactions (Borner et al. 1994).

NMR-spectroscopy and X-ray crystallography (Muchmore et al. 1996) have solved the structure of Bcl-X_L. The tertiary structure of Bcl-X_L bears a resemblance to that of some bacterial toxins, notably diphtheria toxin (Muchmore, Sattler, Liang, Meadows, Harlan, Yoon, Nettesheim, Chang, Thompson, Wong, Ng, & Fesik 1996). These toxins can form pores in outer membranes of cells and translocate proteins to the interior (Parker & Pattus 1993). This has suggested an idea that Bcl-X_L and related proteins may also form membrane pore. However, Bcl-2 family members have only been shown to form membrane pores under non-physiological conditions of pH and tonicity (Minn et al. 1997)

Intrafamily Protein interaction

Interaction between Bcl-2 family members are often described as dimerisations, this may suggest that the complex in which the Bcl-2 family members are is limited to two proteins. There is actually no evidence that this is the case and complexes of Bcl-2 family members may well be made up of more than two proteins. There are several other proteins (not part of the Bcl-2 family) that have been reported to bind to members of the Bcl-2 family. They include r-Ras

(Fernandez-Sarabia & Bischoff 1993), Bag-1 (Takayama et al. 1995) and Nip-1, Nip-2, Nip-3 (Boyd et al. 1994) and any of them could equally well be part of a multi-protein complex containing several molecules of the Bcl-2 family.

Functionally the interaction between anti-apoptotic and pro-apoptotic Bcl-2 members partly dictates their overall effect on apoptosis. This does not however, tell us which subgroups of Bcl-2 family members regulate the activity of others. Are the pro-apoptotic members essential for apoptosis in mammalian cells? Do anti-apoptotic proteins promote cell survival by titrating the pro-apoptotic members or are other proteins (e.g. Ced-4 like proteins) their principal targets? The discovery of mutants of anti-apoptosis family members that cannot bind to pro-apoptotic members but still retain their survival function in mammalian cells makes the model in which the anti-apoptotic members are the crucial regulators of apoptosis mostly likely.

Function

All Bcl-2 proteins presently known have either anti or pro-apoptotic activity and antagonism has been shown to be a general feature of the relationship between the members of these two subfamilies (Oltvai & Korsmeyer 1994).

The prototype Bcl-2 family member is Bcl-2 itself; it was the first to be discovered and is presently the best characterised. Functional studies have been carried out in a number of cell lines. In all these analyses, over expression of Bcl-2 protects cells from apoptosis but does not promote cell proliferation. In cell lines that were growth factor-independent, Bcl-2 antagonised apoptosis induced by exposure to a broad range of cytotoxic conditions, such as Gamma-radiation, UV-

radiation, glucocorticoids, heat shock and oxidative stress (Miyashita & Reed 1992;Tsujimoto 1989;Walton et al. 1993). This indicates that independent signal transduction routes to apoptosis converge upon the final common cell effector machinery that is antagonised by Bcl-2. Over expression of other anti-apoptotic members of the Bcl-2 family have been shown to have functionally indistinguishable effects from that of Bcl-2, indicating that these proteins have similar, if not identical biochemical action (Boise et al. 1993;Chao et al. 1995a;Gibson et al. 1996).

Bax plays a similar representative role for the pro-apoptotic members. It was the first pro-apoptotic protein to be discovered, and is presently the best characterised. Overexpression of Bax results in greatly reduced resistance to cytotoxic stress in many cell lines, and rapid death in others (Oltvai, Milliman, & Korsmeyer 1993). The effects of Bax *in vitro* can be overcome by simultaneous overexpression of Bcl-2 or bcl-xL. Studies using transgenic mice that express Bcl-2 and Bcl-X_L, show that transgene expressing cells from these animals are resistant to the apoptosis inducing effects of a wide range of insults (Chao et al. 1995b;McDonnell et al. 1989;McDonnell et al. 1990;Strasser et al. 1990a). This suggests a final common pathway to death, controlled by the Bcl-2 family members. The ability of Bcl-2 to protect cells from death induced by withdrawal of growth stimuli has made Bcl-2 transgenic mice important research tools in their own right. This has enabled researchers to investigate which physiologically induced cell losses occur by a Bcl-2-inhibitable death programme and has facilitated further study of populations of cells that would be otherwise lost to apoptosis.

The precise mechanisms of action of the Bcl-2 family are not known with certainty. There have been a number of biochemical mechanisms put forward, many of these models have since been questioned. Recent studies have placed the mitochondria at the centre of the decision making step in apoptosis. In this model, it is suggested that the release of apoptogenic proteins such as cytochrome c and apoptosis initiating protein (AIP) from the intermembrane space of the mitochondria triggers the activation of caspases, and constitutes a point of no return in apoptosis. Bcl-2 family members are known to interact with pores in the mitochondria and interfere with the release of these proteins (Hockenbery, Nunez, Milliman, Schreiber, & Korsmeyer 1990;Reed 1997a;Vander Heiden et al. 1999a).

1.2.4.1 Bcl-2 Family Proteins as Determinants of Chemoresponses and Chemoresistance

A variety of investigations have shown that essentially all currently available anticancer drugs induce tumour cell death by triggering apoptosis (Eastman 1993;Patel, Gores, & Kaufmann 1996;Reed 1994;Reed 1995a).Thus, while specific chemotherapeutic agents may have unique primary mechanisms by which they damage tumour cells, presumably they all share the ability to activate cell suicide pathways that culminate in apoptotic death.

Elements of the cell death pathway upon which anticancer drugs depend for killing tumour cells have been conserved throughout evolution. Components of this pathway have been identified, for example, in the free-living nematode, *C. elegans*.

Because Bcl-2 is a blocker of programmed cell death, over expression of Bcl-2 or its related anti-apoptotic homologs prevent or markedly delay the normal cell

turnover in vivo, thus contributing to neoplastic growth by prolonging cell survival rather than by accelerating cell division. The relevance of dysregulated cell death to human cancers can be readily appreciated by consideration of the follicular non-Hodgkin's B cell lymphoma, in which the *bcl-2* gene was first discovered because of its involvement in t(14;18) chromosomal translocation that fuses the *bcl-2* gene on chromosome 18 with the immunoglobulin gene heavy chain locus on chromosome 14 resulting in the overexpression of *bcl-2* (Tsujimoto et al. 1985;Tsujimoto & Croce 1986). The low-grade follicular lymphoma represents the most common type of non-Hodgkin's lymphoma, with ~20,000 new cases annually in the United States alone. Cell cycle analysis of these tumors has shown that the malignant cells are almost entirely G_0/G_1- phase resting B cells, which accumulate in patients not because they are dividing more rapidly than normal but they are failing to turnover at appropriate rates by programmed cell mechanism (Reed, J C. Encyclopedia of cancer. Vol. 1, 3 vol. San Diego: academic press, 1997:125-143). The ability of overexpression of Bcl-2 to produce abnormal expansion of the resting B cells by prolonging cell survival has been confirmed in transgenic mouse experiments, where Bcl-2 expression was driven under the influence of B cell-specific immunoglobulin heavy –chain enhancer elements (McDonnell, Deane, Platt, Nunez, Jaeger, McKearn, & Korsmeyer 1989;McDonnell, Nunez, Platt, Hockenberry, London, McKearn, & Korsmeyer 1990).

Overexpression of Bcl-2 contributes not only to the origins of cancer but also to the difficulties in treating it. This is because Bcl-2 can block or markedly impair the induction of apoptosis by current anticancer drugs. In experiments where gene transfer methods have been used to produce elevations in the expression of Bcl-2 protein, it has been shown that Bcl-2 renders various types of tumor and leukemic cell lines resistant

to the induction of apoptosis by a wide variety of anticancer drugs (Fisher et al. 1993a;Kamesaki et al. 1993;Tang et al. 1994). Similar data have recently been obtained for Bcl-X_L, a close cousin of Bcl-2 that also suppresses apoptosis (Ibrado et al. 1996b;Ibrado et al. 1996a;Minn et al. 1995). These findings further strengthen arguments that currently existing chemotherapuetic drugs have activation of apoptosis as their final common mechanism of cytotoxicity and suggest that Bcl-2 and its homologs regulate a distal step in the evolutionarily conserved cell death pathway (Reed 1994).

Several groups have performed experiments using antisense technology, demonstrating that downregulation of Bcl-2 protein levels can reverse chemoresistance, rendering malignant cells more sensitive to the cytotoxic effects of conventional anticancer drugs. For example, antisense oligonucleotides and expression plasmids have been shown to markedly increase the sensitivity of human B cell lymphoma cell lines to drugs such as dexamethasone, Ara-C and methotrexate (Kitada et al. 1994). Similarly, bcl-2 antisense can increase the sensitivity of freshly isolated AML blasts to Ara-C (Campos et al. 1994). Bcl-2 antisense oligomers improved the cytotoxic response of human prostatic cell lines to VP16 (etoposide). Recently, there have been studies that employ mitochondrial targeting agents that are capable of restoring sensitivity to anticancer drugs by functionally antagonizing the effect of Bcl-2 and related antiapoptotic proteins at this organelle (Larochette et al. 1999;Marchetti et al. 1997;Vos, Grant, & Budke 1976).(Hirsch et al. 1998a) Taken together these findings strongly suggest that pharmacological agents that impair Bcl-2 function could have a major impact on the treatment of cancer. By removing or blocking Bcl-2 as a barrier to apoptosis, theoretically, it should be possible to enhance the apoptotic signals generated

by currently available anticancer therapies to much more effectively drive tumor cells through the cell suicide process.

There is no evidence overexpression of Bcl-2 has any effect on the entry of drugs into tumor cells. Drugs are still able to interact with their primary molecular targets in Bcl-2 overexpressing cells, including damage to DNA or other macromolecules, and cause cell cycle arrest. Bcl-2 therefore defines a novel type of drug-resistance mechanism, one which is distinctly different from other classical mechanisms of chemoresistance involving problems with drug accumulation in tumor cells (mdr-1), amelioration of drug induced damage (DNA repair enzymes) or reduced amounts of drug induced injury (glutathione overproduction). Rather than blocking the primary injury mediated by anticancer drugs, the Bcl-2 family proteins prevent drug-induced damage from being effectively translated into signals for cell death (Reed 1995b). For this reason, Bcl-2 essentially converts anticancer therapies from cytotoxic to cytostatic. Since tumor cells that express Bcl-2 are not readily killed and therefore remain viable, the idea is that these cells may have opportunities to attempt repair of drug-induced damage after drugs are withdrawn or to develop additional secondary genetic changes that result in acquired, classical drug resistance through some of the mechanisms mentioned above.

1.2.4.2 Dysregulation of Bcl-2 protein Production in Human Malignancies

Though the *bcl-2* gene was first discovered in B cell lymphomas, high levels of Bcl-2 protein production have been reported in carcinoma of the prostate, colorectal adenocarcinomas, small cell and non small cell lung cancers, and many other solid

tumor and hematological malignancies (Campos et al. 1993a) . In some cases, these changes in Bcl-2 expression appear to occur as early events in the progression to cancer. In colorectal adenocarcinoma Bcl-2 protein production is found along the crypt-villous axis in early stage adenomatous polyps. In prostatic cancer Bcl-2 expression is associated with the progression to androgen-independent, metastatic disease. Based on the currently available results, it can be estimated that Bcl-2 production occurs in about one-half of all human cancer, suggesting that the dysregulation of programmed cell death as a result of changes in the expression of Bcl-2 represent a common step in human carcinogenesis.

Unlike the chromosomal translocation seen in B cell lymphoma, the *bcl-2* gene is not grossly altered in its structure in solid tumors and most types of leukemias, and the mechanisms responsible for high levels of *bcl-2* gene expression remain largely unknown. One potential explanation, however, is the loss of p53 function that is estimated to occur in about half of human cancers. In this regard, the tumor suppressor gene has been shown to function as a repressor of bcl-2 gene expression in some types of tumor cell lines in vitro and some tissues in vivo (Miyashita et al. 1994;Rampino et al. 1997). Loss of p53 may represent one mechanism that contributes to *bcl-2* gene deregulation in cancer, by relieving *bcl-2* from the transcriptional repression of the wild-type p53 protein. The connection between p53 and regulation of *bcl-2* further emphasizes the important role that *bcl-2* plays as a determinant of chemoresponse, given that p53 has been shown to have a major influence on chemo-and radioresponse of tumors and is of prognostic significance for patients with several types of cancer.

Abnormally high levels of Bcl-X_L protein have been detected in various types of cancer, including poorly differentiated colonic adenocarcinoma and gastric cancers, as

well as advanced prostate adenocarcinomas (Krajewska et al. 1996a;Krajewska et al. 1996b;Rampino, Yamamoto, Ionov, Li, Sawai, Reed, & Perucho 1997). The molecular explanation for these changes in the expression of these Bcl-2 family proteins has not yet been determined.

1.2.4.3 Prognostic Significance Of Bcl-2 Family proteins In Cancer

Bcl-2 has been shown to be of prognostic significance in patients with some type of cancers, including patients with lymphomas, leukemias and prostate cancer in which high levels of bcl-2 expression have been associated with poor responses to chemotherapy, shorter disease-free survival, faster times to relapse, shorter overall survival or other end points that generally are associated with poor clinical outcome (Campos, Rouault, Sabido, Oriol, Roubi, Vasselon, Archimbaud, Magaud, & Guyotat 1993a;Hermine et al. 1996a;Hill et al. 1996;McDonnell et al. 1992;Rampino, Yamamoto, Ionov, Li, Sawai, Reed, & Perucho 1997).

In other cancers, however, Bcl-2 expression has not correlated with poor clinical outcome and has even been paradoxically associated with favourable outcome for patients. Recent studies on other members of the Bcl-2 protein family, particularly Bax, however, suggest a potential explanation for these seemingly paradoxical observations.

Most studies have focused exclusively on Bcl-2. In fact it is the ratio of anti-apoptotic proteins, such as Bcl-2, to pro-apoptotic proteins, such as Bax, that defines the relative sensitivity or resistance of tumour cells to apoptotic stimuli, such as chemotherapeutic drugs and radiation. Consequently, tumour cells can also

become resistant to therapy by reducing their expression of Bax, as opposed to increasing their levels of Bcl-2. This is precisely what has been recently observed for metastatic breast cancer and progressive chronic lymphocytic leukaemia. Moreover reductions in the levels of Bax proteins have been reported in about one-third of breast cancers and have been associated with poor responses to therapy, faster time to tumour progression and shorter overall survival in women with metastatic disease who were treated with combination chemotherapy. Interestingly, these same tumours with reduction in Bax also had reduced Bcl-2 proteins levels, potentially explaining why reduced Bcl-2 has been previously associated with unfavourable outcome for some subgroups of women with breast cancer. Thus, the reduced levels of Bcl-2 were offset by reductions in Bax, presumably resulting on balance in a net survival advantage for these chemoresistant cancers. In this regard, reduced levels of Bax proteins in human breast cancer lines have also been associated with increased resistance to a variety of apoptotic stimuli. Moreover, gene transfer –mediated increases in Bax in human breast cancer cell lines have been reported to restore sensitivity to apoptosis and to impair tumour formation in SCID mice.

Factoring in the Bcl-2 /Bax ratio may also explain some other clinical observations that have previously been difficult to reconcile with Bcl-2's documented function as a suppressor of apoptosis induced by chemotherapeutic drugs and radiation. For example, despite the high levels of Bcl-2 protein found in the 85% of follicular B cell lymphomas as the result of t(14;18) chromosomal translocations, most patients with this disease respond well, at least initially, to treatment and can be induced into a partial or complete remission. Recently,

however, it has been shown that genotoxic stress stimulates marked increases in Bax protein production in lymphoid cells in vivo in normal and in Bcl-2 transgenic mice, presumably because of the ability of p53 to directly transactivate the Bax gene promoter. Thus, by inducing increases in Bax protein levels, genotoxic stress produced by DNA- damaging drugs or radiation may partially overcome the high levels of Bcl-2 caused by t(14;18) translocations in follicular lymphomas.

Still another possible explanation for paradoxical associations of Bcl-2 with better clinical outcome in some types of cancer may be attributable to compensatory increases in Bcl-X_L. For example, in colorectal cancers, Bcl-2 tends to be present at high levels in early-stage well-differentiated tumours but often declines during progression to more aggressive undifferentiated tumours. In contrast to the well-differentiated tumours, these undifferentiated colorectal tumours tend to express Bcl-X_L at high levels rather than Bcl-2. Similar observations have made for gastric carcinomas. Moreover, it has been reported that Bcl-xl levels become elevated during progression of prostate cancers to high grade primary (Gleason stage 8-10) and metastatic disease. Thus, Bcl-xl may substitute for Bcl-2 in some types of advanced cancers.

Taken together, these observations illustrate the complexity of attempting to predict clinical outcome based on measurements of Bcl-2 alone and emphasize the importance of systematically screening tumours for alterations in the expression of other members of the Bcl-2 family. Each particular type of cancer may have a certain member of the Bcl-2 family that predominates in terms of its prognostic power. The challenge then is to determine which members of the Bcl-2 family are the most relevant to predicting responses to chemotherapy or overall

survival in specific subgroups of patients. It is also important to bear in mind that all efforts to utilize Bcl-2 family proteins as prognostic indicators have relied upon sampling the tumours at one particular time prior to institution of therapy. However, the regulation of the levels of Bcl-2 and its homologs is likely to be a dynamic process in many tumours, as illustrated by the effects that p53 can have on expression of Bax and Bcl-2. Thus, there exist inherent limitations in the approaches that are typically taken for assessing the value of Bcl-2 family proteins as prognostic indicators.

1.2.4.4 Potential Functions Of Bcl-2 Family Proteins

The biochemical mechanism by which Bcl-2 and its relatives regulate cell death remains unknown to date. The predicted amino-acid sequences of Bcl-2 and its homologs, as deduced from cDNA cloning, share no significant homology with other proteins that have a defined biochemical or enzymatic function. Thus, it has been a challenge to understand in biochemical terms how Bcl-2 family proteins modulate cell life and death.

In the simplest sense, Bcl-2 family proteins can be thought of as regulators of a distal step in the cell death pathway. The idea is that multiple stimuli that initiate the cell death process funnel their signals somehow through a final common pathway or pathways that are regulated, at least in part, by Bcl-2 and its homologs. The available information suggests that Bcl-2 family proteins somehow either amplify or suppress these poorly defined signalling events that initiate apoptosis, with the relative ratios of the proapoptotic and the antiapoptotic members of the family dictating whether cells remain viable or trigger the

apoptotic cell death machinery when confronted with a given cell death signal. The primary effectors of apoptosis appear to be a family of cysteine proteases that cleave their target proteins after asparatic acid residues ("caspases" for cysteine asparatic acid proteases) (Alnemri et al. 1996). The caspases are produced as inactive zymogens (proproteins) in cells and become activated by proteolytic cleavage. Overexpression of Bcl-2 or Bcl-X_L, for example, prevents the cleavage and activation of various caspases under circumstances where these Bcl-2 family proteins prevent apoptosis (Boulakia et al. 1996;Ibrado, Huang, Fang, Liu, & Bhalla 1996b;Monney et al. 1996;Smyth et al. 1996). Conversely, overexpression of Bax induces processing and activation of certain members of the caspases family (Jurgensmeier et al. 1997). This relation of Bcl-2 to the proteases has its roots in the genetic analysis of cell death in the nematode, C. *elegans*, where it was first established that the worm homolog of Bcl-2 (ced-9) suppresses the function of the worm caspases. (Ced3) (Hengartner & Horvitz 1994a;Hengartner & Horvitz 1994b).

Several theories have been advanced as to how Bcl-2 and its homologs control cell life and death. Certainly, Bcl-2 family proteins could potentially regulate the caspases directly, though to date there is no support for this idea. Alternatively Bcl-2 may act as an anti-oxidant pathway in cells (Hockenbery et al. 1993;Kane et al. 1993;Shimizu et al. 1995). It has also been suggested that Bcl-2 may regulate the homeostasis of Ca^{2+} in cells, based on experiments, which have shown an ability of Bcl-2 overexpression to (a) influence the sequestration of Ca2+ within the ER, preventing its releases into the cytosol during apoptosis and delaying release induced by thapsigargin, a specific inhibitor of the ER's Ca^{2+}-

ATPase; (b) prevent entry of Ca^{2+} into the nucleus after treatment of cells with thapsigargin or chemotherapeutic drugs; (c) inhibit the release of Ca2+ from mitochondria after exposure of cells to uncouplers of oxidative phosphorylation and (d) potentiate maximal Ca^{2+} uptake capacity of mitochondria (Baffy et al. 1993;Lam et al. 1994;Marin et al. 1996). Evidence has also been presented suggesting that Bcl-2 can control the transport of proteins across biological membranes, particularly the nuclear envelope (Marin, Fernandez et al. 1996;Meikrantz et al. 1994;Ryan et al. 1994). In this regard, electron microscopic studies have demonstrated the presence of Bcl-2 protein in association with what appear to be nuclear pore complexes (Krajewski et al.1993a). Finally, the association of the Bcl-2 with the kinase Raf-1 and possibly with the GTPase R-Ras has raised the possibility that Bcl-2 may control a signal transduction pathway that is focused on the intracellular membrane compartments where Bcl-2 resides rather than the plasma membrane where such enzymes are associated with growth factor receptor (Fernandez-Sarabia & Bischoff 1993;Wang et al. 1994;Wang et al. 1995). However, a direct cause-and-effect relationship has not been demonstrated between Bcl-2 and these processes, and at this point we do not know whether the effects of Bcl-2 on the redox state, Ca^{2+} compartmentalization, protein transport and protease activation represent direct effects of Bcl-2 versus downstream events that are hundreds of steps removed from Bcl-2.

1.2.4.5 Bcl-2 Family Proteins As Regulators Of Mitochondrial Permeability Transition

Recently, evidence has been accumulating that Bcl-2 family proteins can regulate the phenomenon of permeability transition (PT) in mitochondria. PT results from the opening of a large megachannel located at the contact sites in mitochondria

where the inner and outer membranes abut and where various transport processes involving ions and proteins occur. It can be measured in isolated mitochondria based on an increase in their light-absorbance caused by mitochondria swelling in isotonic medium or by the failure of such mitochondria to take up cationic fluorescent dyes which rely upon an intact electrochemical gradient for entry into mitochondria. Interestingly, electron microscopic studies suggest that Bcl-2 is concentrated at these contact sites in the outer membrane of mitochondria (de Jong et al. 1994).

The biochemical composition of the megachannel is poorly defined at present, but among its components appears to be the voltage-dependent anion channel, the peripheral benzodiazepine receptor (mBzR) and porin are located in the outer membrane, while the adenine nucleotide translocator is located in the inner membrane (de Jong, Prins, Mason, Reed, van Ommen, & Kluin 1994) A mitochondria-specific cyclophilin located in either the intermembrane space or the matrix also interacts with the megachannel and cyclosporin analogs that inhibit its peptidylproyl cis-transisomerase activity can prevent the induction of PT under some circumstances (Nicolli et al. 1996).

The induction of PT results in several potentially lethal events in cells, including (a) dissipation of the electrochemical gradient (mitochondrial membrane potential $\Delta\Psi m$) and a subsequent shutdown in oxidative phosphorylation, resulting secondarily in the generation of reactive oxygen species (ROS) a consequence of the interrupted flow in the electron transport chain at the level of cytochrome c; (b) dumping of Ca2+ from mitochondria into the cytosol; and (c) release of mitochondrial proteins into the cytosol (Igbavboa, Zwizinski, & Pfeiffer 1989;Marchetti et al. 1996a). Among the proteins released are cytochrome c and an unidentified ~50 kDa apoptogenic protein

termed AIF for Apoptosis Inducing Factor (Liu et al. 1996;Susin et al. 1996a). Both cytochrome c and AIF reside normally in the intermembrane space of the mitochondria. When added to cytosolic extracts derived from healthy nonapoptotic cells, both cytochrome c and AIF can induce rapid activation of ICE-family proteases (caspases) and trigger apoptosis-like destruction of native nuclei added to these extracts (Liu et al. 1996;Susin et al.1996a).Thus, with effects on ROS, Ca2+ and caspase activation, if Bcl-2 does regulate mitochondrial PT, then this could unify several of the phenomena described for Bcl-2 into a single mechanism.

The evidence that Bcl-2 can regulate PT comes from experiments using both intact cells and isolated mitochondria. In intact cells, a wide variety of apoptotic insults can induce mitochondrial PT, as defined by reduced uptake of cationic fluorescent dyes such as $DiOC6_{(3)}$ and rhodium 123 into mitochondria and subsequent generation of reactive oxygen species (Marchetti et al. 1996a;Zamzami et al. 1996a). Overexpression of Bcl-2 prevents the loss of electrochemical gradient across mitochondria and suppresses the subsequent production of ROS and release of apoptogenic proteins under conditions where Bcl-2 also prevents apoptosis, but not when the apoptotic stimulus is such that Bcl-2 fails to prevent cell death (Susin et al. 1996b). In experiments employing isolated mitochondria where the mitochondria are derived from Bcl-2 overexpressing cells or their control transfected counterparts that have low levels of Bcl-2, it has been shown that Bcl-2 can prevent the induction of PT by oxidants, Ca2+ and atractyloside (an inhibitor of the adenine nucleotide translocator). Though examined only recently within the context of apoptotic cell death, mitochondrial PT has been studied for decades with regards to mechanisms of necrotic cell death particularly during ischemia and reperfusion injury (Bernardi, Broekemeier, & Pfeiffer 1994). It is

tempting therefore to speculate that the ability of Bcl-2 to prevent PT induction by a wide variety of insults, including elevated cytosolic Ca^{2+} and oxidative injury, may provide an explanation for reports that demonstrate that Bcl-2 can also prevent necrotic cell death under some circumstances (Kane et al. 1995).

Major questions at this point for the field of apoptosis research are whether all the effects of Bcl-2 can be explained by regulation of mitochondrial PT and whether all pathways to apoptosis go through a mitochondria-dependent step. The finding that Bcl-2 protects against cell death in cells that lack mitochondrial DNA (rho-zero cells) and that are therefore incapable of oxidative phosphorylation at first glance appears to argue against the PT hypothesis (Jacobson et al. 1993), but even cells that cannot execute oxidative phosphorylation maintain an electrochemical gradient across their mitochondria due to reverse function of the ADP/ATP antiporter, which under times of anaerobic metabolism transports ATP made in the cytosol from glycolysis into the mitochondria for sustenance of various mitochondrial functions that are essential for cell viability. Moreover, even rho-zero cells can be induced to undergo apoptosis in association with triggering of mitochondrial permeability transition (Marchetti et al. 1996b).

However, it has also been shown that Bcl-2 can suppress apoptosis induced by some types of stimuli in certain types of cells when targeted to the endoplasmic reticulum by replacement of the usual C-terminal membrane-anchoring domain of Bcl-2 with an ER targeting transmembrane domain from cytochrome b5 (Zhu et al. 1996).Though it is possible that a small portion of this chimeric Bcl-2/cyto-b5 protein manages to find its way onto the mitochondria surface, this result suggests that Bcl-2 may have other functions besides inhibiting

mitochondrial PT. Moreover, the aforementioned experiments demonstrating effects of Bcl-2 overexpression on Ca2+ and protein transport across the nuclear envelope also speak of potential non-mitochondrial functions for Bcl-2.

1.2.4.6 Bcl-2 Family Proteins As Channel Formers

A milestone in our understanding of Bcl-2 family protein function has come from the three-dimensional structure of the Bcl-X_L protein, which has revealed striking structural similarity with the pore-forming domains of the bacterial toxins, diphtheria toxin (DT) and the colicins (Muchmore et al.1996). The structures of Bcl-X_L and the pore forming domains DT and colicins consist entirely of helices connected by variable length loops. Each structure contains a pair of core hydrophobic helices that are long enough to penetrate the lipid bilayer and which are shielded from the aqueous environment by the other five to seven amphipathic helices that orient their hydrophobic surfaces towards the central core helices and their hydrophilic surfaces outward. Studies of the bacterial toxins suggest that under conditions of low pH, acidic lipid membranes and creation of a voltage gradient, the central hydrophobic helices efficiently insert through the lipid bilayer as one step in the process of pore-formation (Cramer et al. 1992;Duche et al. 1996;Merrill, Cohen, & Cramer 1990;Parker et al. 1992;Parker & Pattus 1993). True to its structural similarity to these bacterial toxins, recent data indicate that recombinant Bcl-X_L protein, as well as Bcl-2 and Bax, can form pores in liposomes in a PH and acidic lipid membrane-dependent fashion (Minn et al.1997;Schendel et al. 1997). Moreover, single channel recordings in planar bilayers, which provide an exquisitely sensitive method for monitoring pore formation, indicate that even at neutral PH, Bcl-2, Bcl-X_L and Bax can form discrete ion-conducting channels.

Since both anti-apoptotic (Bcl-2; Bcl-X_L) and pro-apoptotic (Bax) proteins are capable of forming channels in membranes, it remains unclear at present how this pore-forming activity relates to the bioactivities of these proteins. In addition, many other issues remain unresolved, such as the diameter of the channels and how many Bcl-2, Bcl-X_L or Bax proteins it takes to create an aqueous channel in membranes. In the case of DT, the channels are evidently large enough to transport a protein, since the primary function of DT is thought to be transport of the ADP-ribosylation factor subunit of the toxin from lysosomes and endosomes into the cytosol (Donovan et al. 1981). In contrast, the bacterial colicins transport ions (Cramer et al.1992;Konisky 1982). With respect to some of the cellular phenomena that Bcl-2 family proteins have been reported to control such as mitochondrial PT, transporting either ions (such as Ca2+) or proteins (such as cytochrome C and AIF) could fit nicely with the structural and electrophysiological evidence of pore formation. Indeed, it can even be argued that Bcl-2 family proteins create the mysterious mega channel that causes mitochondrial PT. Arguing against this possibility however are data indicating that even yeast mitochondria exhibit mega channel behavior, and yet no homologs of Bcl-2 evidently exist in yeast.

1.2.4.7 Strategies For Inhibiting Bcl-2 Function in Cancer

To a large extent the failure to find cures to cancer lies with the lack of any new drugs that attack the problem in truly novel ways. Essentially all anticancer drugs currently available to clinical oncologists target some aspect of the cell division machinery in an effort to arrest the growth of dividing cells. These agents, for example, may target enzymes required for nucleotide precursor synthesis, induce damage directly to DNA in

an effort to slow DNA-replication and lethally damage cells, or interfere with the function of microtubules thereby arresting cell division during mitosis. While the mechanisms by which the currently available anticancer drugs arrest cell proliferation are well understood, it has only been in recent years that the molecular details of how anticancer drugs actually kill tumor cells have begun to be delineated and the central role that apoptosis plays in this process appreciated.

The central role that Bcl-2 family proteins play as regulators of a distal step in this pathway positions them as ideal targets for impacting cancer therapy in truly significant ways. Several strategies can be envisioned for abrogating the effects of Bcl-2 or other anti-apoptotic members of the Bcl-2 family, such as Bcl-X_L in human cancers. These include small molecules blockers of Bcl-2/Bax and Bcl-X_L/Bax dimerization, as well as blockers of the interactions of Bcl-2 with accessory proteins, such as BAG-1 and Raf-1, that promote cell survival in collaboration with Bcl-2. As the pore like functions and biological relevance of Bcl-2 family proteins are better understood (Boise et al.1993;Boise, Gottschalk, Quintans, & Thompson 1995;Chao, Linette, Boise, White, Thompson, & Korsmeyer 1995b;Halestrap et al. 2000;Jacobson 1997;Minn et al.1997), there is a growing interest in a new class of molecules that interact with these proteins either directly or indirectly altering the function of these proteins at various points within the cell such as the mitochondria as a means of enhancing apoptosis (Hortelano et al. 1997;Petronilli et al. 1994a;Reed 1997b).

1.3 Apoptosis and Cholangiocarcinoma

That Abnormalities in the Bcl-2 protein family are involved in the development of cancer is well established. Bcl-2 itself was originally isolated as a proto-oncogene (Cleary, Smith, & Sklar 1986;Tsujimoto et al. 1984), and transgenic mice that overexpress Bcl-2 are more prone to tumourogenesis that controls. On its own Bcl-2 is a weak transforming oncogene, but it synergises potently in lymphomagenesis with growth promoting oncogenes, such as *c-myc* and *prim-1*, in doubly transgenic mice (Acton et al. 1992;Strasser et al. 1990b). This has led to the conclusion that Bcl-2 overexpression functions in neoplastic transformation by extending the life span of cells, thereby facilitating the acquisition of further oncogenic mutations. Such a process has been suggested to occur in the pathogenesis of cholangiocarcinoma (Sikora & Kapoor 1999). Studies have shown that cholangiocarcinoma cells possess growth promoting genes such as k-ras (Imai et al. 1996;Lee et al. 1995;Ohashi et al. 1994) and c-erb-2 (Chow et al. 1995) along with expression of Bcl-2 (Charlotte et al. 1994a;Skopelitou et al. 1996a).

Anticancer treatments such as chemotherapy and radiotherapy induce apoptosis in sensitive cells. Oncogenes and tumour suppressor genes, which regulate cell death, influence the response of tumour cells to anti-cancer therapies. Cancer cells that are resistant to apoptosis respond poorly to these forms of cancer treatment (Dive 1997a). The poor response of cholangiocarcinoma to chemotherapy and radiotherapy (Bukowski, Leichman, & Rivkin 1983a;Leung & Kuan 1997a;Pitt et al.1995;Sahin 1997;Urego, Flickinger, & Carr 1999), may in part be explained by an inability of apoptosis induction by such cytotoxic agents.

There is very little evidence to date to substantiate such a hypothesis in the case of cholangiocarcinoma, however, in other solid and haematological malignancies reports are emerging to support a role of the Bcl-2 family members in the susceptibility of tumour cells to cytotoxic therapy induced cell death (Campos et al. 1993b;Friedman et al. 1997;Hermine et al. 1996b;Leung & Kuan 1997a). Low levels of spontaneous and induced apoptosis in cholangiocarcinoma cells may partly explain the poor response rate observed following treatment with radiotherapy and/or chemotherapy.

AIM

This study investigated the co-expression of the antiapoptosis proteins Bcl-2, Bcl-X_L and Mcl-1 in cholangiocarcinoma cells. Using functional anatagonists of these proteins their effects on cholangiocarcinoma cell apoptosis following chemotherapy and radiotherapy was also investigated both *in vitro* and *in vivo*.

Chapter 2 Materials and Methods

In this section the experimental techniques are described which are common to more than one chapter in this thesis. For experimental technique(s) performed in a single chapter only, the description was restricted to the relevant chapter.

2.1 Tissue Culture

The culture of animal cells and tissue is now a widely used technique in many different disciplines from the basic sciences of cell and molecular biology to the rapidly advancing field of biotechnology.

2.1.1 Biology of cells in culture

The list of cell types which can now be grown in culture is quite extensive, and includes connective tissue elements such as fibroblasts, skeletal tissue (bone and cartilage), cardiac and smooth muscle, epithelial tissue (liver, lung, breast, skin, bladder and kidney), neural cells, endocrine cells (pituitary, adrenal, pancreatic islet cells), melanocytes, and many different types of tumours.

The use of markers that are cell type specific has made it possible to determine the lineage from which many of these cultures were derived, but what is not clear, in many cases, is the position of the cells within the lineage. In a propagated cell line, a precursor cell type will predominate, rather than a fully differentiated cell, which would not normally proliferate. Consequently the cell line

may be heterogeneous for example cultures of epidermal keratinocytes, contain stem cells, precursor cells, and keratinized squames. Culture heterogeneity also results from lineages being present in the cell line. The only unifying factors are the selective conditions of the media and substrate, and the predominance of the cell type (or types) that have the maximum growth rate. This tends to select the common phenotype, but due to the cell interactive nature of growth control, may obscure the fact that the population may contain several distinct phenotypes only detectable by cloning.

Because the dynamic properties of cell culture are sometimes difficult to control, and the appropriate cell interactions found *in vivo* are difficult to recreate *in vitro*, many people have forsaken the idea of serial propagation in favour of retaining the structural integrity of the original tissue. Such a system is called histotypic or organ culture. Attempts have also been made to recreate tissue-like structures *in vitro* by re-aggregating different cell types and culturing at high density as multicellular spheroids (Freyer & Sutherland 1980), perfused multilayers on glass or plastic substrates (Kruse, Jr. & Miedema 1965).

Tissue culture is a generic term that encompasses organ culture, where a small fragment of tissue or whole embryonic organ is explanted to retain tissue architecture and cell culture (cell line) where the tissue is dispersed mechanically or enzymatically, or by spontaneous migration from an explant, and may be propagated as a cell suspension (non-adherent) or attached layer (adherent line).

Cell lines are devoid of structural organisation, have lost their histotypic architecture and often the biochemical propagation associated with it, and generally do not achieve a steady state unless special conditions are employed. They can,

however, be propagated and hence expanded and divided into identical replicates, they can be characterised and a defined cell population preserved by freezing

2.1.2 Human Cell lines and Tissue Culture Techniques

There are a limited number of human cholangiocarcinoma cell lines commercially available for use in the study of this disease. Most of the widely used cell line banks such as European collections of cells cultures (ECACC) do not stock human or animal derived cholangiocarcinoma cell lines. Through the kind efforts of the staff at ECACC, it was possible to locate one centre in Europe that stocked two human cholangiocarcinoma cell lines Tfk-1 and Egi-1. In addition, other cell lines were studied to analyse and compare *in vitro* responses to chemotherapy, theses included the bcl-X_L expressing human leukaemic cell lines BV173 (Solary et al. 1996), the Bcl-2 expressing lymphoma cell line SUDHL4 (Parker et al. 1998)and the colorectal cell line HT29. These cell lines were obained from ECACC. The human cholangiocarcinoma cell lines used in this study are:

1. Egi-1 (Scherdin et al. 1987)
2. Tfk-1 (Saijyo et al. 1995)

These well characterised human cell lines were all derived from patients prior to exposure to either chemotherapy or radiotherapy. All cell lines were maintained in culture and used for experiments at the tissue culture laboratories of both the Royal Free and the Royal London Hospitals. The cell culture and experimental procedures were all performed with the strict adherence to the COSHH guidelines and departmental safety policies. Details of the protocols for culture medium

preparation, growth, subculture and storage, all performed within a laminar flow hood are described below:

2.1.2.1 Culture Medium

In line with the suppliers recommendations BV173, Sudh-L4, Tfk-1 and HT29 were cultured at 37°c with 5% CO_2 in RPMI 1640 medium (Sigma, Dorset, UK) supplemented with 5mM glutamine, 10% Foetal calf serum (FCS) and 100U/ml penicillin 100ug/ml streptomycin, while Egi-1and Sk-Cha-1 were grown in 1:1 Minimum Essential Medium (MEM) and Dulbecco's Modified Essential Medium (DMEM) (Sigma, Dorset, UK) supplemented with 1mM non-essential amino acids, 2mM essential amino acids, 5mM glutamine, penicillin-streptomycin supplements and 10% FCS. This provided the optimum growth conditions for each of the cell lines. Cells were incubated in approximately 5mls and 15mls culture medium in $25cm^2$ and $75cm^2$ vented flasks respectively. Culture medium in healthy growing cultures was changed every 48 to 72 hours in order to prevent nutrient exhaustion and cellular waste overproduction.

2.1.2.2 Growth and subculture

Protocols for the thawing out and growth supplied by the suppliers were followed; aliquots once starting to thaw were immediately diluted with warm culture medium to neutralise the dimethyl-sulphoximide (DMSO). Cells were then plated out in $25cm^2$ vented flasks and placed in the incubator. Culture medium was changed after 24 hours, by which time all viable cells would have adhered to the bottom of the plate allowing for the removal of all dead floating cells and cellular debris.

Culture conditions and medium change were performed in the usual manner until pre-confluence (exponential growth phase) at which point 1:5 subculture (splitting) was performed using 1x trypsin/EDTA (Sigma, Dorset, UK). All cells lines had an approximate doubling time of 40 hours.

2.1.2.3 Storage

2 x 10^6 of exponentially growing cells were counted using the haemocytometer for freeze storage in a 5ml mixture made of 70% culture medium, 20% FCS and 10% (DMSO). Well labelled and dated aliquots of cells were immediately placed within alcohol bathed holding devices in the –70°c freezer to ensure gradual freezing for 48 hours before being transferred into liquid nitrogen containers at a temperature of –270°c.

2.1.2.4 *In vitro* experiments on cell lines

All the *in vitro* experiments were performed in 6-12 well flat-bottomed plates in triplicates. Following a series of control experiments to assess the approximate number of cells to be seeded at the start of the 96 hour experiment time scale, it was found that 0.25 x 10^5 cells per well was optimum as this number of cells failed to reach confluence within 96 hours, hence still within the exponential gowth phase. This was necessary in order to prevent either overcrowding (growth arrest) by the 4^{th} day or poor growth due to lack of appropriate cell to cell interaction. During the 96 hour period cells were fed once at 48 hours by addition of 3mls warmed culture medium.

Experiments began the evening before with the splitting, counting and plating out of cells in accurately labelled and dated flat-bottomed experimental plates. This allows trypsinized cells to adhere to the bottom of the plate overnight. The following morning usually at 0845 hours all the wells containing cells being used in the experiment had the culture medium changed, to provide the cells with fresh medium for optimal growth and also to remove any dead unattached cells that may interfere with later analysis. At set time intervals 0, 24, 48, 72 and 96 hours experiments were terminated. Each well was treated in an identical manner. The culture medium containing dead and dying (early apoptotic) cells from each well were collected in an accurately labelled FACS tube. The cells were spun down to a cell pellet, then collected and added to trypsin detached counterparts from the corresponding well for incubation with mitochondrial dyes and propidium iodide prior to FACS analysis.

2.2 Immunohistochemistry

This staining method is used to localise (useful) tissue antigens (proteins) by using specific immunoglobulins (antibodies) against the antigen(s) of interest. The method utilises an enzyme-substrate reaction to convert a colourless chromogen into a coloured end product.

2.2.1 Antibody

There are five classes of immunoglobulins (IgG, IgM, IgA, IgD and IgE). By far IgG and IgM are most frequently used in immunohistochemistry. Depending on the method of production antibodies can be polyclonal or monoclonal.

Different cells produce polyclonal antibodies and, in consequence, are immunochemically dissimilar; they react with various epitopes on the antigen against which they are raised. The most frequently used animal for polyclonal antibody production is the rabbit. The antigen in question is frequently injected intradermally or subcutaneously. Booster doses are usually given to maintain antibody levels. Polyclonal antibodies can be obtained in the form of whole stabilised antiserum or fractions purified to varying degrees, after the cells are removed from the blood harvested from the animal. The antigen-specific antibody desired is isolated by affinity chromatography.

Clones of plasma cells fused with myeloma cell lines produce monoclonal antibodies. Antibodies from a given clone are immunochemically identical and react with a specific epitope on the antigen against which they were raised. Mice are commonly used for production of monoclonal antibodies. After the immune response has been archieved, B-lymphocytes from the spleen or lymph node are harvested and fused with nonsecreting mouse myeloma cells (hybridoma) under specific conditions for longevity.

There are numerous advantages of monoclonal antibodies in immunohistochemistry over their polyclonal counterparts; these include high homogeneity, absence of nonspecific antibodies, ease of characterisation and no batch-to-batch variability. However, there are some pitfalls in the use of

monoclonal antibodies such as, the methods for the screening of useful clones and for quality control must be identical to the methods for which the antibodies were raised. All too often, monoclonal antibodies are characterised using frozen tissue, for example, when they are intended for use on formalin-fixed specimens. Survival of the target epitope following fixation is important. In some instances, the target antigen survives fixation (by use of polyclonal antibodies), but the particular epitope with which the monoclonal reacts may not.

2.2.2 Staining Methods

There are many immunoenzymatic-staining methods that can be used to localise antigens. The choice is based on the individual needs of each laboratory, such as type of specimen being investigated, the degree of sensitivity required, and the processing time and cost requirement. The following are some examples of methods of staining.

Direct method

In this technique an enzyme labelled primary antibody reacts with the antigen in the tissue. Subsequent use of substrate and chromogen concludes the reaction. Because this method utilises only one antibody, it can be completed quickly, and nonspecific reactions are limited. However, since staining involves only one labelled antibody, little signal amplification is archieved. This method is now rarely used.

Indirect methods

Two-step: In this method an unconjugated primary antibody binds to the antigen. An enzyme-labelled second antibody against the primary antibody (now the antigen) is then applied, followed by the substrate-chromogen solution. If the primary antibody is made in rabbit or mouse, the second antibody must be directed against rabbit or mouse immunoglobulins, respectively. This method is more versatile than the direct method because a variety of antibodies from the same species can be used with the same-labelled secondary antibody. The procedure is also several times more sensitive than the direct method because several secondary antibodies are likely to react with different epitopes on the primary. As a consequence, this attaches more enzyme molecules at the site of antigen and hence results in greater sensitivity. Undesired reaction may occur if the second antibody reacts with endogenous immunoglobulins in the specimen. Using preabsorbed antiserum, that is, secondary serum, which has been absorbed with immunoglobulin from the species from which the specimen was obtained, however, can prevent this cross-reactivity.

One of the most frequent applications of the indirect technique is the detection of autoimmune antibodies in human serum. In this case, the patient's serum is the primary antibody and is applied to a tissue containing the antigen under study. An enzyme-linked secondary antibody to human immunoglobulin is added. If the serum contains antibodies to the antigen, the enzyme-linked secondary antibody will bind to the patient's antibody and thereby localise the antigen.

Three-step method: In the three step indirect method, a secondary enzyme-conjugated antibody layer is added to the previously described indirect technique.

The primary and enzyme-conjugated secondary antibody is applied sequentially, followed by a third enzyme-conjugated antibody specific to the secondary antibody. For example, if the secondary antibody were made in goat, the third antibody must be specific for goat immunoglobulin. Both secondary and tertiary antibodies must be conjugated to the same enzyme. The addition of the third layer of antibody serves to further amplify the signal, since more antibodies are capable of binding to the previously bound secondary reagent. This places additional enzyme at the site of the tissue antigen and thereby creates better colour intensity. The enhanced signal is particularly helpful when staining antigens with limited number of epitopes. This method provides a simple way to increase staining intensity. The sensitivity of this technique provides a good alternative to soluble immune complex and avidin-biotin procedures.

Soluble Immune Complex Method

This method is sometimes also called the unlabelled antibody method and utilises a preformed enzyme anti-enzyme immune complex. This consists of an enzyme (the antigen), and the antibody directed against the enzyme. To obtain a soluble enzyme-antienzyme complex, the enzyme is added in excess and any precipitate is removed.

The staining sequence is as follows: unconjugated primary antibody, secondary antibody, soluble enzyme-antienzyme complex, and substrate solution. The primary antibody and the antibody of the enzyme immune complex must be made in the same species so that the secondary antibody can link the two together. The secondary antibody, also called the link antibody, has to meet two requirements:

first, it must be directed against immunoglobulins of the species producing the primary antibody and enzyme immune complex; second, it must be added in excess so that one of its Fab sites binds to the primary antibody leaving the other Fab site free to bind antibody from the enzyme immune complex. Soluble enzyme-antienzyme immune complex techniques are named after the particular enzyme immune complex they use. For example, the PAP method utilises a peroxidase-antiperoxidase complex, APAAP uses an alkaline phosphatase-antialkaline phosphatase complex, GAG uses glucose oxidase-antiglucose oxidase and so forth. The most commonly used methods are PAP and APAAP. The PAP complex consists of three molecules of peroxidase and two molecules of alkaline phosphatase and one antibody against the enzyme. Soluble immune complex methods are among the most sensitive immunochemical techniques. The technique makes use of the natural affinity of antibody for antigen by using a stable immune complex as opposed to the harsher chemical conjugation process. The considerably greater degree of sensitivity compared to the previously described methods is mainly attributable to more enzyme molecules being localised per antigenic site.

The enzyme-antienzyme complex method gives excellent results on fixed, paraffin-embedded specimens. The PAP method is a commonly used technique for demonstrating tissue and cell antigens by light microscopy. The APAAP method allows the staining of tissues rich in endogenous peroxidase. It is particularly useful for blood smears where quenching with hydrogen peroxide denatures the leukocyte antigens.

Avidin-Biotin Method

These methods utilise the high affinity of avidin or streptavidin, for biotin (dissociation constant 10^{-19}M). Avidin has four binding sites for biotin. However, due to the molecular orientation of the biotin-binding sites, fewer than four molecules of biotin will actually bind. Currently two avidin-biotin methods are in frequent use- the avidin-biotin complex (ABC) method and the labelled avidin-biotin (LAB) technique. Both methods require a biotinylated antibody as a link antibody. Biotinylation is a mild process, whereby biotin is covalently attached to the antibody. Open sites on avidin form the avidin-biotin complex or enzyme-labelled avidin bind to the biotin on the link antibody. The biotinylated antibody does not have to be added in excess since free Fab sites are not needed for binding. The sequence of reagent application is primary antibody, biotinylated secondary antibody, preformed avidin-biotin-enzyme complex (ABC) or enzyme-labelled avidin (LAB), and substrate solution. Horseradish peroxidase and alkaline phosphatase are commonly used enzyme labels. The strong affinity of avidin for biotin and the mild biotinylation process make the avidin-biotin methods more sensitive than the previously described direct and indirect methods. The original authors of the ABC method found the procedure to be greater in sensitivity than the PAP method.

Much of the early work with the ABC method focused on the identification of pituitary hormones in normal and neoplastic tissue. Today, both ABC and LAB methods lend themselves to the localisation of numerous antigens in a variety of specimens. As with the immune complex methods, excellent results can be archieved on fixed, paraffin-embedded specimens. Some tissues such as the liver

and kidneys contain endogenous biotin (EABA), which must be blocked to avoid nonspecific staining.

2.2.3 Enzymes

Immunoenzymatic staining methods utilise an enzyme-substrate reaction to convert colourless chromogens to coloured end products. There are several different enzymes such as horseradish peroxidase, glucose oxidase and calf intestine alkaline phosphatase. Selecting the enzyme most suitable for a particular immunohistochemical application depends on a number of criteria:

1. The enzyme should be available in highly purified form and be relatively inexpensive.
2. Conjugation (covalent binding to antibody or avidin, for example) or non-covalent binding should not abolish enzyme activity, although it may diminish it.
3. The bound enzyme should be stable in solution.
4. Endogenous enzyme activity should interfere only minimally with specific antigen-related staining.
5. The products of the enzymic reaction should be readily detectable and stable.

Horseradish peroxidase

This enzyme (molecular weight 40 kD) is isolated from the root of the horseradish plant. Peroxidase has an iron-containing heme group (hematin) as its active site and in solution is coloured brown. The hematin of the peroxidase first forms a complex with hydrogen peroxide and then causes it to decompose resulting in water and atomic oxygen. Peroxidase oxidises several substances, two of which

are polyphenols and nitrates. The complex formed between peroxidase and excess hydrogen peroxide is catalytically inactive and in the absence of an electron donor (e.g. chromogenic substance), is relatively inhibited. It is the excess hydrogen peroxide and the absence of electron donor that brings about quenching of endogenous peroxidase activities. Cyanide and azide are two other strong (reversible) inhibitors of peroxidase. Peroxidase can be attached to other proteins either covalently or noncovalently.

Calf Intestinal Alkaline Phosphatase

Calf intestinal alkaline phosphatase (molecular weight 100kD) removes (by hydrolysis) and transfers phosphate groups from organic esters by breaking the P-O bond; an intermediate enzyme-substrate (PO_4) bond is briefly formed. The chief metal activators for alkaline phosphatase are Mg^{++}, Mn^{++} and Ca^{++}.

The unlabelled anti-alkaline phosphatase procedure (the APAAP procedure), which uses soluble immune complexes, molecular weights of around 560kD, has an advantage over the PAP technique (which also uses soluble immune complexes) in that there is no interference from endogenous peroxidase. The APAAP technique is particularly recommended for use on blood and bone marrow smears.

Glucose Oxidase (*Aspergillus niger*)

This enzyme (molecular weight 185kD) has been found in several molds. Since its prosthetic group is composed of flavin, it is referred to as a flavoprotein (flavoenzyme). Flavin undergoes reduction by accepting hydrogen from the oxidation of glucose and passing it on to a receptor, such as oxygen. There is no

glucose oxidase in mammalian tissue, so no endogenous enzyme activity is encountered. Glucose oxidase has been utilised in labelled and unlabelled antibody techniques, as well as in avidin-biotin technology. The chief disadvantage of glucose oxidase is its relatively low sensitivity when compared to peroxidase and alkaline phosphatase. Because of the low sensitivity glucose oxidase requires 10 fold higher concentrations of primary antibody.

2.2.4 Substrates and Chromogens

Peroxidase activity as described above, in the presence of an electron donor, results in the formation of an enzyme-substrate complex. The electron donor provides the "driving" force in continuing catalysis of H_20_2, while its absence effectively stops the reaction.

There are several electron donors, which, upon being oxidised, become coloured products and are therefore called chromogens. This, and the property of becoming insoluble upon oxidation, makes such electrons useful in immunohistochemistry.

Three, 3'-Diaminobenzidine Tetrahydrochloride (DAB) produces a brown end product, which is highly insoluble in alcohol and other organic solvents. Oxidation of DAB also causes polymerisation, resulting in the ability to react with osmium tetraoxide, and thus increasing its staining intensity and electron density. Of the several metals and methods used to intensify the optical density of polymerised DAB, gold chloride in combination with silver sulfide appears to be the most successful (Newman, Jasani, & Williams 1983).

Other chromogens used include:

Three-Amino-9-Ethylcarbazole (AEC) upon oxidation, AEC forms a red rose colour that is alcohol soluble. Therefore, specimens processed with AEC must not be immersed in alcohol or alcohol solutions. Instead an aqueous counterstaining and mounting should be used. To avoid further oxidation upon exposure to light, slides stained with AEC should be stored in the dark.

Four-Chloro-1-Naphtol (CN) precipitates as a blue end product. Unlike DAB, CN tends to diffuse from the site of precipitation.

2.2.5 Controls

Reagent and procedure controls are necessary for the validation of immunohistochemical staining results. Without their use, interpretation of staining, in itself a subjective art, would be hazardous, and results would be of doubtful value.

Reagent controls

This is to ascertain whether the primary and secondary antibodies are specific for their target antigens.

Procedure Controls

Procedure controls serve to ascertain primarily whether the staining protocols were followed correctly, whether day-to-day and worker-to-worker variations have occurred, and whether reagents continue to be in good working order. Procedure controls involve reagent substitution and tissue controls.

Reagent substitution Of all the components used in an immunohistochemical staining system, the primary antibody is, without a doubt, the most critical,

although occasionally other reagents may need to be replaced. To ascertain the specificity of the primary antibody, the primary antibody should be replaced with either the affinity-absorbed antiserum, with another irrelevant antibody, or with preimmune or nonimmune serum from the same species that produced the primary antibody. When the primary antibody is monoclonal, use of an irrelevant antibody is probably the best negative reagent control. The paramount objective in the selection of a good control in all cases is to limit all facets of the primary antibody except the antigen specificity. Not only should normal serum or nonimmune IgG replace the whole antiserum or its IgG fraction, respectively, but also the IgG fractions should contain nearly identical protein fractions.

Tissue Controls

Tissue controls can be either negative, positive controls or internal type.

Negative Tissue controls; These specimens are processed (fixed, embedded) identically to the unknown, but do not contain the relevant tissue marker.

Positive Tissue Controls; These controls are processed identical to the specimens and contain the target protein. In some cases, it will be advantageous to have this control tissue stain marginally positive, so as to monitor not only for the presence of the antigen, but also any loss of sensitivity. The latter would be particularly important when staining tumour, for example. In this case, staining intensity may vary with the degree of tumour differentiation.

Internal Positive Control tissue This type of control, also known as "built-in" control, is ideal because the variables of tissue preparation, processing, and staining are eliminated. Built-in controls contain the target marker in the tumour to be identified and adjacent normal elements. One example is the presence of S-

100 protein in melanoma and normal tissue elements, such as peripheral nerves and melanocytes. Built-in controls have the additional advantage that no separate positive control sections are required.

The three-step avidin biotin complex method for antigen localisation with DAB as the chromogen was the immunohistochemical method used in this study as described below :

Sequential tissue sections (3μm) were mounted on 3-Aminopropyl-Triethixy-Saline (APES, Sigma, Dorset, UK) coated slides. Paraffin embedded samples were 1st dewaxed by passing through graduated washes in xylene, before being dehydrated in absolute alcohol. Endogenous peroxidase was blocked by immersion for 10 minutes in 3% H_2O_2, after which slides were washed in running water. Antigen retrieval was performed by microwaving (800W, full power) in citrate buffer (pH 6-7) for 20 minutes before bathing in water for 2 minutes. A protein block step was achieved by incubation in 10% normal goat serum for 10mins.

Using the three-step streptavidin-biotin complex method Bcl-2 staining was as follows; slides were incubated with anti-human Bcl-2 primary monoclonal antibody (Dako Ltd, Ely. UK) at 1:50 or negative mouse IgG1 (control antibody) (Dako, Ltd. Ely. UK) at 1:400 at room temperature for 1 hour. Secondary (1:100) and tertiary (1:100) layers were incubated for 30 minutes each (DAKO ABC kit). For Bcl-X_L and Mcl-1 staining the catalyzed signal amplification system (CSA, K1500. Dako Ltd, Ely. UK) was used. This method also incorporates streptavidin-biotin complex, but has additional steps made up of biotinylated tyramide signal enhancement (recommended by Dako) (Erber, Willis, & Hoffman 1997). The

system was adapted for rabbit primary antibody using the biotinylated anti-rabbit link (Dako Ltd, Ely. UK). Anti-human Mcl-1 (Dako Ltd, Ely. UK) and Bcl-X_L (Autogen Bioclear UK LTD, Wiltshire.UK) primary polyclonal antibodies were incubated with slides through a series of 15 minute steps at room temperature at a dilution of 1:1000 and 1:750 respectively. Normal rabbit Immunoglobulin (Ig) as first antibody layer (serum) formed negative control. To reduce non-specific background staining 0.1% tween 20 (w/v) and 0.1% bovine serum albumin (w/v) were added to tris buffered saline (TBS, pH 7.6). This solution was routinely used to dilute the antibodies and wash the sections. In addition the sections were bathed in the background reducing solution for 5 minutes between each step during the staining process. Slides were then developed using 3,3-diaminobenzidine tetrahydrochloride (DAB) for 10 minutes followed by counterstaining with haematoxylin. Slides were examined by light microscopy and the number of cells staining positive was assessed with the aid of the corresponding H&E slide.

2.3 Immunofluorescence

The principles of Immunofluorescence are identical to immunohistochemistry. The only difference being that the primary (one-step) or the secondary (two-step) antibody has an attached Fluorescein isothiocynate conjugated (FITC) molecule to enable detection by flow cytometry. In this study the one step method was used for immunodetection of relevant proteins. The reason for the use of this identical method of protein detection in this study was to allow for the more accurate subcellular localisation, a feature of relevance in the study of the Bcl-2 family proteins. The technique is described below

The adherent cholangiocarcinoma cell lines Tfk-1 and Egi-1 were trypsinized pre-confluence using trypsin/EDTA (x 1, Sigma, Dorset.UK). After washing in phosphate buffered saline, cells were fixed and permeabilised using the Dako intrastain kit (Dako Ltd, Ely. UK). Fluorescein isothiocynate conjugated (FITC) rabbit anti-human Mcl-1and Bcl-X_L polyclonal antibodies and FITC monoclonal anti-human Bcl-2 antibody (Dako Ltd, Ely. UK) were incubated at 1 in 100 dilution in the dark at room temperature for 15 minutes. Negative isotype controls (rabbit Ig) were used at the appropriate dilution. Cells were then mounted and cover slipped. For p-gp analysis cells were prepared as described above but were not permeabilised before incubation with the antibody. Images were either captured using a Zeiss Axioskop fluorescent microscope in conjunction with IPlab spectrum image analysis software (version 3.1.1) or analysed by flow cytometry

2.4 Flow Cytometry

Flow cytometry enables scattered fluorescent light from individual cells in fluid suspension to be quantified at very rapid speeds. Typically up to 5000 cells can be measured per second. The quantitative aspects of the technology take their origins from the work of Caspersson and colleagues in the 1930s, where stained images were projected on to a wall and the amount of light absorbed in different areas of the images was quantified with primitive photodetectors.

The technology has a number of advantages and disadvantages. The former includes objective quantification of specific molecules, statistical precision, multi-parametric cross-correlated data analysis, distribution information and hence subset identification, dynamic measurements, sensitivity, speed and the generation of vast amounts of data. The disadvantages include loss of "geographical"

information from solid tissues as a single cell-suspension is mandatory, absence of a direct visual record, and the generation of vast amounts of data. The last point is included under both headings as it is a two-edged sword. Data are meaningless until converted to information, and this may present considerable problems, particularly for multi-parametric data.

The technique relies upon measuring both scattered light and fluorescence from suitably stained constituents in individual cells in the population. The stained cells are streamed in single file in fluid suspension through the focus of a high intensity light source. As each cell passes through the focus, a flash scattered and/or fluorescent light is emitted. This is collected by lens systems and filtered before reaching a photodetector that may be either a photomultiplier or a solid-state device. The photodetector quantitatively converts the light flash into an electric signal, which is digitised by an analogue-to-digital converter into a whole number (integer) that is then sorted electronically. The data can subsequently be recalled for display and analysis. Modern machines are more "user friendly" with built-in computers, which run most of the tasks that in old machines had to be done manually.

2.4.1 Principles of Operation

The most essential feature of any flow cytometric instrument is a stable fluid stream, which presents the cells one at a time to a sensing volume where the measurements are made. To obtain consistency of measurement, each cell must be presented in the same volume within the sensor. This is achieved by hydrodynamic focusing, a concept which stems from the work of Bernoulli who showed that pressure and velocity are inversely related in fluid flow, and Euler who showed

that velocity profile is parabolic with the greatest velocity in the centre of the stream. Thus, the pressure is lowest in the centre so any non-compressible particles will tend to flow coaxially in the centre where the pressure is lowest. The flow chamber consists of a closed cylinder with an inlet port, forming a sheath through which fluid is pumped, and an exit constriction. Cells are introduced into the flow by a needle whose tip is located just above the exit constriction. The combination of hydrodynamic focusing and the coaxial pressure drops causes cells to pass down the centre of flow through the exit nozzle. By suitably adjusting the nozzle size, the constriction cone, flow rates, and relative pressures it is possible to constrain one cell at a time to pass through the nozzle, with the supporting medium containing the cells making up the core of the coaxial stream in the nozzle. Single-sheath systems can attain positional accuracy of the core to within +/-2μm, which is adequate for most applications.

The other requirements are a high-intensity light source to elicit fluorescence and light scatter, suitable light collection optics, and electronics to quantitate the response from individual cells.

2.4.2 Excitation

Due to the relatively small number of fluorescent molecules per cell and the short time each cell is exposed to the exciting light (1-5μs), it is necessary to archive very high light fluxes at the intersection of the cell stream with the illumination. A high light flux means that a very large number of photons are passing through a small volume of space, and this requires that the source of the illumination must be as small as possible and as bright as possible. The best illumination source is

undoubtedly the laser for several reasons. Firstly, the light is very bright. Secondly, the beam emitted by a laser is coherent, which means that the light is polarised, the photons are all "in step", and the beam is essentially parallel. The last attribute means that effective spot size of the illumination source is very small, tending to a point, because the parallel beam appears to be coming from infinity. Finally, lasers give a more stable output than conventional sources, which is essential for hours of continuous operation.

2.4.3 Application of Flow cytometry in this study

The ability for flow cytometry to analyse large quantities of cells with rapid speed and precession, with the added advantage of reproducibility made the use of this technique helpful in the vast amounts of kinetic studies required in this thesis. With the aid of different protocols for cell preparation and fluorescent probes it was possible to use flow cytometry to acquire data for different components of this study. Below is an outline of some of the uses.

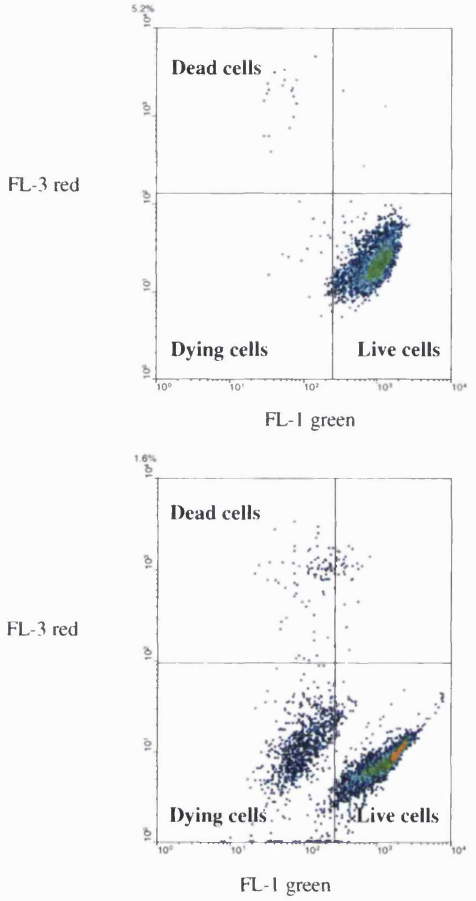
Mitochondrial transmembrane potential Measurement ($\Delta\Psi m$)

Mitochondria are vital cellular components in multicellular organisms responsible for the generation of the majority of the energy required to perform vital cellular functions. Not surprisingly any process that is capable of interfering with mitochondrial energy production will have serious implication on cell survival. A potential gradient called the transmembrane potential gradient ($\Delta\Psi m$) exists between the intermembrane space and the mitochondrial matrix. $\Delta\Psi m$ is required

for the normal function of mitochondria. The gradient is created and sustained by the exchange of proton involved in the generation of ATP by the FiFo ATPase (Bernardi, Broekemeier, & Pfeiffer 1994;Bernardi & Petronilli 1996). This gradient can be measured by virtue of the fact that when present, certain fluorescent molecules (probes or dyes) are preferentially concentrated (taken up) in the mitochondria, resulting in the emission of a higher mean fluorescent intensity (MFI), than when there is loss or dissipation of this gradient ($\Delta\Psi m$). This drop in the uptake (MFI) is easily measurable using flow cytometry (figures 2a and b). Following the discovery that the mitochondria played a key role in the regulation of apoptosis, studies into the changes in structure and physiology of this organelle have shown that early in the process of apoptosis there is a loss or dissipation of $\Delta\Psi m$. This event is now widely accepted to represent an irreversible commitment to apoptosis thus providing an additional measurable parameter in apoptosis (Marchetti et al.1996a;Marchetti et al. 1996c;Petit et al. 1996;Zamzami et al. 1996b). In this study loss of $\Delta\Psi m$ formed one of the parameters for measuring apoptosis at single cell level using the mitochondrial potentiometric dye $DiOC6_{(3)}$, in combination with propidium iodide as described below.

Mitochondrial dysfunction, one of the parameters for detecting apoptosis was measured at single cell level by detecting the collapse of the inner mitochondrial membrane potential, $\Delta\Psi_m$ (Cai, Yang, & Jones 1998;Hirsch et al. 1573;Hirsch et al. 1998b;Marchetti et al. 1995). The $\Delta\Psi_m$ was measured by flow cytometry along the FL1 and FL3 channels acquiring 10,000 events (cells) per well (figure 2a and 2b). Trypsinised cells re-suspended in culture medium for analysis were incubated for 15 minutes in the dark at room temperature with the

potentiometric probe 75nM 3, 3 dihexyloxalocardocyanine ($DiOC6_{(3)}$) and 20μg/ml propidium iodide (PI). $DiOC6_{(3)}$ concentation within the mitochondria is determined by the potential gradient that exist across the mitochondrial membrane. When cells begin to undergo apoptosis this gradient ($\Delta\Psi m$) is lost (mitochondrial permeability transition- MPT) which results in the lower uptake of the green dye $DiOC6_{(3)}$. To adequately measure this early stage in apoptosis it is necessary to exclude from the analysis those cells that are already dead using the red dye propidium iodide (PI) which fluoresces along the FL3 channel. Using these two dyes (multi-paramentric) together creates three different cell populations, the green dye high + red dye low group of normal live cells, the green dye low + red dye low group of early apoptotic cells and the green dye low + red dye high group of apoptotic or dead cells. All the data collected was analyzed using software package WinMDI 2.8 windows multiple document interface for flow cytometry (TSRI, La Jolla, California, USA) illustrated in figures 2a, b and c. This was then exported into a microsoft excel sheet for chart generation and statistics

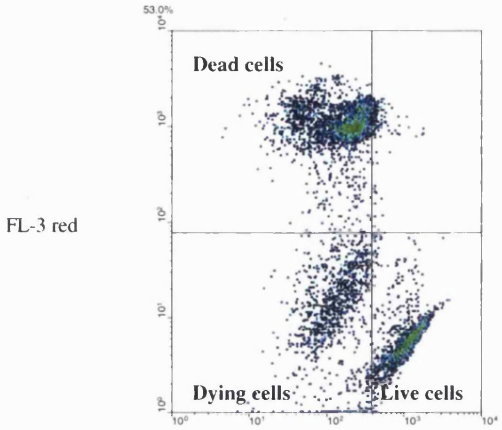


(Live cells - $DiOC6_{(3)}$ high/PI low, Dying cells $DiOC6_{(3)}$ low/PI low and dead cells $DiOC6_{(3)}$ low/PI high)

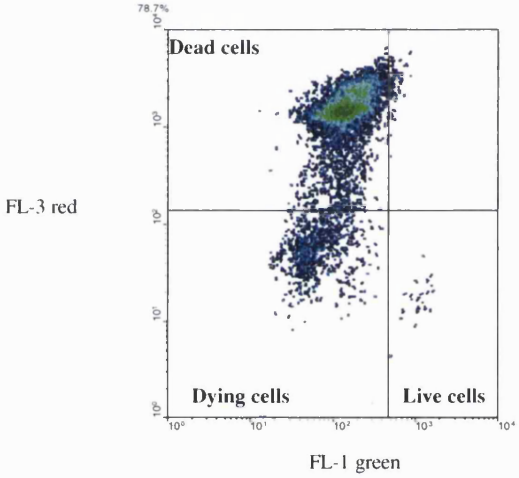
Figure 2a

Density plot of FL-1 green channel (X axis) against FL-3 red channel (Y axis) demonstrating the drop in the green dye uptake ($DiOC6_{(3)}$, 1 log drop in MFI) that occurs with permeability transition (loss of $\Delta\Psi m$). The control sample is featured in the upper plot (A), 24 hours after stimulation. As the cells begin to undergo apoptosis they loose the $\Delta\Psi m$ as shown in the lower plot (B) as cells are still alive and have intact plasma membranes they have a low uptake of propridium iodide (red) (dying cells).

A



B



(Live cells - $DiOC6_{(3)}$ high/PI low, Dying cells $DiOC6_{(3)}$ low/PI low and dead cells $DiOC6_{(3)}$ low/PI high)

Figure 2b

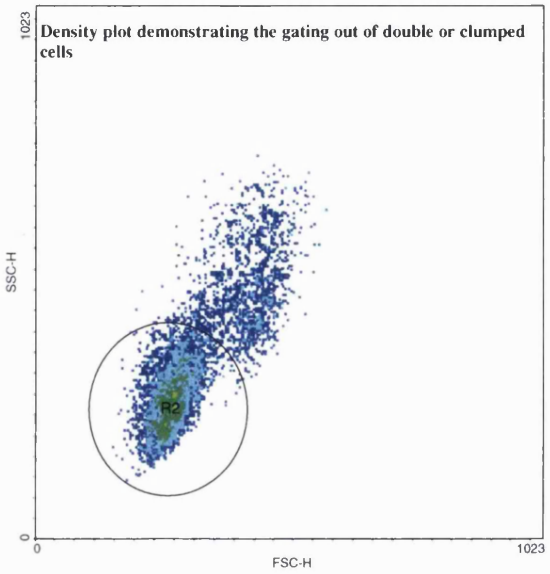
Density plots of FL-1 against FL-3 channels demonstrating the changes in red dye (propidium iodide) uptake in the $DiOC6_{(3)}$ low cells (dying) that occurs with the loss of plasma membrane integrity shown here 48 (upper) and 72 hours (lower) after treatment. N.B there is a 2 log increase in the uptake of the red dye (dead) as seen here on the FL-3 channel.

DNA staining (cell cycle analysis)

The cell cycle responses were analysed in each group. Cells to be analysed were collected by trypsinization. Cells were re-suspended in 100ul of phosphate buffered saline (PBS) and spun down to a pellet before being permeabilised by re-suspending in 70% ethanol on ice (4°C) for 30 minutes. Cells were then washed three times in PBS and resuspended in 500ul of PBS containing 50ug/ml propidium iodide (PI) (Sigma Ltd) and RNAse A (Sigma Ltd) at 200ug/ml for 30 minutes at room temperature.

Integral channel fluorescence can be plotted against peak channel fluorescence on the Becton Dickinson cytometer. Singlet diploid cells lie on a 45° line from bottom left of the plot to top right. A gate round these cells excludes doublet cells or cells clumped together. Cells in G_0/G_1 phase of the cell cycle with a normal DNA content (figure 2c), group towards the bottom left of the plot, cells in G_2M lie towards the top right of the plot and cells in S phase lie between these extremes. Apoptotic cells with a DNA content less than G_0G_1 congregate below and to the left of the G_0G_1 population. A gate around all these gives an apoptotic cell population to the left of the G_0G_1 peak when displayed on a more conventional histogram plot. The percentage of apoptotic cells and cells in the various stages of the cell cycle can be quantified by placing additional gates on the appropriate histograms of the DNA profile (figure 2c).

A



B

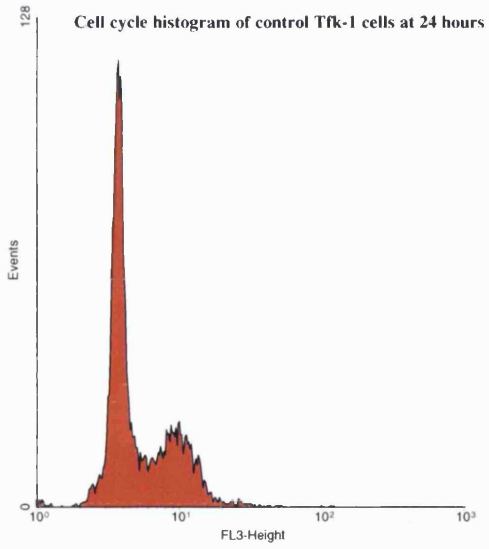


Figure 2c

Density plot of forward scatter (FSC) against side scatter (SSC) (upper) demonstrating the position of the gate (R2) in order to excludes double or clumped cells from the analysis along with a histogram showing the cell cycle mapping of normal cells during their exponential growth phase (lower)

Protein detection by fluorescein isothiocynate conjugated antibodies (FITC)

Using FITC conjugated antibodies cell surface and cytoplasmic antigens were investigated as described above.

2.5 Cytotoxicity Studies (Inducers of Apoptosis)

A wide variety of stimuli including chemotherapeutic drugs, UV and X-ray irradiation are used to induce apoptosis in living cells of both human and animal origin. Dose response studies, can be performed *in vitro, in vivo* and *ex vivo*. This approach and adaptations of it has over the years provided scientists with information that has proven beneficial to the study and understanding of cancer cell biology and development of novel cytotoxic agents. Despite significant advances in the overall understanding of tumour cell genetics and apoptosis, differences at just about every level exist between different cancers making it necessary for the study of specific tumour types. There have been very few studies to date focusing on the responses of cholangiocarcinoma *in vitro* to cytotoxic therapy. Clinical data suggests an overall poor response to therapy. There is a need to study this type of tumour in vitro to understand its apoptotic responses that may pave the way for the development of novel strategies aimed at improving treatment. The kinetics of apoptosis of the human cholangiocarcinoma cells was studied following exposure to a number of cytotoxic drugs, UV and X-ray irradiation.

2.5.1 Cytotoxic Drugs

5 flourouracil (5FU) the antimetabolite is the 1st line drug of choice used in both single agent and combination chemotherapy in Cholangiocarcinoma (Oberfield &

Rossi 1988). Other agents that have been used to treat this disease include gemcitabine (Poplin et al. 1999b) antibiotic mitomycin c and the microtubule disrupting agent paclitaxel (Hidalgo et al. 2001;Murren et al. 2000). For the dose response studies single agent drugs, 5FU and gemcitabine were used. In addition the topoisomerase 2 inhibitor etoposide (VP16) was studied because it has a well documented apoptosis inducing effect (Forbes et al. 1992;Holm et al. 1994;McGahon et al.;Nakajima et al. 1994). None of the drugs used are known to be substrates for the multi-drug resistant protein transport system. The single drug concentrations used in the sensitisation experiments was derived from the dose response studies. 10μM was the dose of choice as both human cell lines responded only partially after 96 hours to either 5FU or etoposide.

2.5.2 UV Irradiation

UV irradiation is a recognised inducer of apoptosis *in vitro*, it has the advantage of being both easy to administer and reproducible. Cholangiocarcinoma cells were exposed for 1,3 and 5 minutes to the apoptosis inducing effects of UV irradiation form source (120mJ/cm^2, Chromato-UV trans-illuminator, model TM-20).

2.5.3 Radiotherapy (X-ray irradiation)

Radiotherapy plays an important part in the treatment of CCA (Karani et al.1985;Milella et al.1998). This form of anticancer therapy can be delivered to tumours externally (tachytherapy) or internally (brachytherapy), or a combination

of both. Total exposure doses range on average from 45-60Gy in 6-8 fractions (Urego, Flickinger, & Carr 1999), which is about equivalent to a single exposure dose of 5-8Gy. For the dose response studies, cells in culture were exposed to a radiation dose of 0.5-10Gy. The set up and exposure protocol used to deliver the radiation is outlined below:

12-well plates containing CCA cells in culture were placed on the couch with the gantry at 0^0. A 1cm thick perspex sheet was then placed on the top and bottom of the plate with the sides of the plates surrounded with padding (bolus). The field setting was 30 x 30 at 100cm SSD from the surface of the perspex. The computer was pre-programmed to deliver between 0.5-10Gy radiation doses to CCA cells *in vitro* from a 6MV linear accelerator (Elekta oncology SL75/5).

2.6 Assessment and measurement of Apoptosis

Cholangiocarcinoma cell undergoing apoptosis was observed using light microscopy of H&E or papaconiclau stained slides (figures 2d).

CHAPTER 3

Expression of antiapoptotic proteins and multidrug resistant (MDR) protein p-Glycoprotein (p-gp) in Human Cholangiocarcinoma

3.1 Introduction

The Bcl-2 family of proteins are important regulators of apoptosis. Following the discovery and cloning of the Bcl-2 gene, there has been unprecedented interest in the process of apoptosis. This has led to significant advances in the understanding of the regulation of apoptosis and the critical role played by the Bcl-2 family in this process. Members of the Bcl-2 family of proteins can now be divided on the basis of their effect on apoptosis into two groups that play opposing roles in the regulation of apoptosis. Bcl-2, Bcl-X_L, Mcl-1, bcl-w, survivin are examples of antiapoptotic members while bax, bid, bcl-X_S and bad are proapoptotic members. Studies involving gene transfer into cell lines, transgenic mice, and EBV infection (Henderson et al. 1991) have demonstrated that the Bcl-2 protein inhibits apoptosis induced by a wide variety of stimuli including chemotherapy and radiotherapy. Bcl-X_L protein has a structure very similar to Bcl-2 and has the ability to prevent apoptosis in an identical way to Bcl-2 (Chao et al.1995a). These proteins through their carboxy-terminus attach to cell membranes of target organelles, such as the endoplasmic reticulum, mitochondrial and nuclear

membranes (Krajewski et al. 1993b). The mitochondria play a central role in the regulation of apoptosis through the release of apoptogenic proteins required for caspase activation (Petit et al.1996), an important and rate-limiting step that is influenced directly by some members of the Bcl-2 family (Reed 1997a;Vander Heiden et al. 1999b;Vander Heiden & Thompson 1999). Members of the family are capable of directly altering the response of both normal and malignant cells to conditions of cellular stress such as growth factor withdrawal, DNA damage and oxidative stress, stimuli that trigger apoptosis (Reed 1997c;Susin et al.1996b;Yang & Korsmeyer 1996). The mechanisms by which members of the Bcl-2 family control apoptosis is now emerging and in part relates to their ability to interact directly and indirectly with the important downstream organelle in apoptosis such as the mitochondria. This important interaction places both the mitochondria and the Bcl-2 protein family in a central position as intracellular regulators in apoptosis. (Crompton 1999) (Kluck et al. 1997;Marchetti et al.1996c;Muchmore et al.1996;Reed 1997c;Susin et al.1996b).

Whether anti-apoptotic proteins expressed by certain malignant cells are capable of altering the response of solid tumours to chemotherapy is unproven. However, in some types of cancer, mostly haematological in origin, the expression of anti-apoptotic proteins is independently associated with a less favourable outcome following chemotherapy (Campos et al.1993b;Hermine et al.1996a;Hill et al.1996;Minn et al.1995). Such observations parallel the findings in experimental models (Kamesaki et al.1993;Kane et al.1993;Lin et al.1996;McDonnell, Deane, Platt, Nunez, Jaeger, McKearn, & Korsmeyer 1989;Miyashita & Reed 1992;Strasser, Harris, & Cory 1991). This would suggest

that malignant cells are equipped with mechanisms that enable them to circumvent the apoptosis inducing effects of chemotherapy and radiotherapy resulting in failure of treatment (Dive 1997a).

The expression of bcl-2 protein by cholangiocarcinoma cells has previously been investigated with conflicting results (Arora et al. 1999a;Charlotte et al. 1994b;Skopelitou et al. 1996b), no previous studies have analysed the expression of the other well characterised antiapoptotic proteins Bcl-X_L or Mcl-1. Whether the reistance of cholangiocarcinoma cells to chemotherapy may be due to the expression of the drug resistant protein p-glycoprotein is unknown.

3.2 Aim

To study the expression of Bcl-2, Bcl-X_L and Mcl-1 proteins in human cholangiocarcinoma archival specimens, human cholangiocarcinoma cell lines and normal biliary eithelium. In addition, the cell surface expression of the MDR protein p-gp was evaluated in human cholangiocarcinoma cell lines.

3.3 Material and Methods

3.3.1 Cholangiocarcinoma Specimens

Cholangiocarcinoma tissue was obtained from the archives of the department of histopathology at the Royal Free Hospital. Tissue specimens held in the department records date back to 1979. The specimens before storage are labelled, indexed and coded, according to histological diagnosis and pathology number, the information is then entered on a computer database (SNOMED).

Using the database, histopathology reports of 30 cases of cholangiocarcinoma were identified and the blocks containing paraffin-embedded material were retrieved using the pathology number, patient and specimen details. In addition, 7 specimens of normal gallbladder were located. The blocks retrieved from the storage rooms, were prepared and cut onto slides as outlined below. From each block a haematoxylin and eosin (H&E) stained slide was prepared and examined by a consultant pathologist in order to verify diagnosis. In addition, fresh normal biliary epithelial specimens were obtained from 10 patients known to have gallstone disease that were undergoing endoscopic retrograde cholangiopancreatography (ERCP). All archival specimens had been fixed in 4% neutral buffered formaldehyde and mounted in paraffin blocks. Each block contained one piece of tissue and was serially sectioned. The H&E stained sections were vital in confirming the diagnosis, identifying and outlining the areas of malignant, normal and dysplastic epithelium.

3.3.2 Preparation of specimens

The paraffin blocks were cooled in an ice bath, and then with the use of a standard microtome cut either 5 μm thick for H&E staining or 3μm thick in sequence for immunohistochemistry. The specimens were floated in a water bath heated to 45°C. The specimens were then mounted on 3-Aminopropyl-Triethixy-Saline (APES, Sigma, Dorset, UK) coated microscopic slides to avoid tissue loss during immunostaining. Slides were labelled at the upper right hand corner of the frosted portion of the slide to ensure correct orientation when handling slides, using a lead pencil, making a record of the respective pathology number. Slides were then allowed to dry in a warm room at 37°C for 24hrs.

Brush and bile samples collected during ERCP were centrifuged at 2800rpm (900g) for 5 minutes, the cell pellet was then re-suspended in RPMI (sigma) and 2 drops of albumin added. 100μl aliquots of the cell suspension were then applied to coated slides by cytocentrifugation at 90rpm for 2 minutes. Slides were then either routinely alcohol fixed and stained with papaconiclau or allowed to air dry before immersion in acetone at room temperature for immunohistochemistry.

3.3.3 Immunohistochemistry

The three-step avidin biotin complex method for antigen localisation with DAB as the chromogen was the immunohistochemical method used in this study as described in chapter 2.

3.3.4 Fluorescence microscopy and Immunofluorescence analysis of Bcl-2, Mcl-1, Bcl-X_L and p-gp

3.5 Results

Results of antiapoptotic protein expression by cholangiocarcinoma are summarised in table 3a

There was no Bcl-2 protein detected in cholangiocarcinoma cells from both paraffin embedded preserved tissue and human cholangiocarcinoma cell lines despite strong positive staining of the internal control areas in the specimens such as lymphoid aggregates and intra-epithelial lymphocytes (Figures 3 a and b). In addition, none of the the human cell lines expressed the cell surface MDR protein p-gp.

The cholangiocarcinoma cells in both specimen groups were found to stain positive for both antiapoptotic Mcl-1 and Bcl-X_L proteins with the majority of the cells showing co-expression of these proteins (figures 3c, d and e). This positive staining occurred though in both the normal and malignant cell populations. The pattern and distribution of the staining was characteristically granular in appearance showing localisation to intracellular organelles such as the mitochondria (figure 3b). Immunofluorescence demonstrated this distribution more clearly (figures 3f).

3.6 Discussion

Cholangiocarcinoma tissue examined in this study had no detectable Bcl-2 protein expression. Expression of Bcl-2 has previously been reported in some cases of cholangiocarcinoma (Charlotte et al. 1994a;Skopelitou et al. 1996a). The reasons for this difference in detection of Bcl-2 in cholangiocarcinoma are unclear. In the previous studies the authors report the use of identical immunohistochemical techniques for protein detection in paraffin embedded samples to those used in the present study. However, Arora *et al* previously reported an absence of Bcl-2 protein in cholangiocarcinoma using immunohistochemistry (Arora et al. 1999b). The explanation for this difference in detection remains unclear. However, considering that Bcl-2 protein was detected in the internal positive controls, the absence of Bcl-2 detection in this study is unlikely to be due to poor antigen retrieval or low Bcl-2 antibody activity. Molecular studies are required to quantify bcl-2 protein expression in cholangiocarcinoma (mRNA). Whether there are other antiapoptotic proteins in the cases where bcl-2 is not expressed that take over the role of Bcl-2 within the cells has yet to be shown. Taking into account the fact that the number of cholangiocarcinoma samples examined in this study (archival tissue from 30 cases and 3 cell lines) makes this the largest single study of Bcl-2 protein expression in human cholangiocarcinoma, along with findings published by Arora and colleagues, it appears that the majority of cases of cholangiocarcinoma studied for the expression of Bcl-2 protein using immunohistochemistry do not express Bcl-2 protein.

Interestingly all the 30 cholangiocarcinoma specimens and the 3 human derived cell lines stained positively for the antiapoptotic proteins Bcl-X_L and Mcl-1. In addition, benign biliary epithelial cells from both preserved normal gallbladder tissue and fresh

samples taken from biliary exfoliative cytology were also found to express Bcl-X_L and Mcl-1. The pattern of staining within the cytoplasm showed these proteins were localised to intracellular organelles especially the mitochondria. The localisation to this important player in the process of apoptosis is vital to their function.

Although a double staining immunohistochemical technique is required to conclusively prove that both Bcl-X_L and Mcl-1 are co-expressed in the same cell, the staining from sequential sections and cell lines for both antibodies strongly suggest this to be the case.

The expression of antiapoptotic proteins Mcl-1 and Bcl-X_L in human cholangiocarcinoma has not been previously reported. This study has demonstrated that these proteins are expressed not only in the malignant but also in the benign biliary epithelial cells. Mcl-1 and Bcl-X_L are important cell survival proteins. It may be that these proteins have a role in cell survival within the biliary tree, perhaps taking over the role played by Bcl-2 protein. There is little known about turnover of cells within the biliary tree, but it is known that bile and its constituents are toxic to hepatocytes, enterocytes and other cells (Mahmoud et al. 1999;Sola et al. 2002;Wilson 2002;Zeid et al. 1997). It is possible that prolonging the survival of these cells through the expression of such antiapoptotic proteins by the cells that line both the intrahepatic and extrahepatic bile duct is necessary to limit excessive epithelial cell loss.

Unfortunately immunohistochemistry is only semi quantitative and should not be used to evaluate differences in protein levels.

This study has demonstrated that human cholangiocarcinoma cells express antiapoptotic proteins, but gives no indication as to whether they are clinically relevant to the resistance of cholangiocarcinoma to chemotherapy or radiotherapy induced apoptosis. In the next phase of this study human cholangiocarcinoma cell lines Egi-1 and Tfk-1 which co-express mitochondrial antiapoptotic proteins Bcl-X_L and Mcl-1 were evaluated for chemotherapy and radiotherapy induced apoptosis.

Chapter 4

The *In Vitro* Apoptotic Responses of Human Cholangiocarcinoma cells to Cytotoxic Therapy

4.1 Introduction

Drug discovery is being transformed by new developments in molecular cell biology and the information sciences. Since formal screening of potential anticancer drugs began around 1955, many thousands of drugs have shown activity in either cell or animal models, but only 39 that are used exclusively for chemotherapy have won approval from Licensing bodies like the U.S Food and Drug Administration. Groups like the NCI have established a primary screen in which compounds are tested *in vitro* for their ability to inhibit growth of 60 different human cell lines (Bader et al. 1991;Smith et al. 1987). This "disease orientated" strategy for drug discovery was based on the hypothesis that selective activity *in vitro* against cancer cell lines from a particular organ would predict selective activity against tumours in humans, a concept that is being tested as agents progress through clinical trails. Patterns of activity observed in cytotoxicity screens, have proved predictive in an even more powerful way at the molecular level, providing valuable information on the mechanism of action of the compounds tested and on molecular targets and modulators of activity within the cancer cells.

There are an increasing number of drugs used mostly under experimental conditions that are broadly described as response modifying agents. These molecules have a number of advantages which include their ease of passage through the cell membrane and pre-determined mode of action to mention a few.

Owing to varying apoptosis thresholds, chemotherapy may induce apoptosis in susceptible cancer cells, but merely a cell cycle pause in their normal counterparts (Dive 1997b;Volm 1998a). Conversely, resistance to anticancer therapies may arises from insensitivity to apoptosis induction (Decaudin et al. 1998;Fisher et al. 1993b;Hickman et al. 1992;Hickman 1996;Hickman & Boyle 1997). Apoptosis resistance is now accepted as an important contributor multi-drug resistance. Many mechanisms of drug resistance have been described including a decreased drug uptake, an increase in DNA damage repair, enhanced drug detoxification, an altered level or mutation of the intracellular drug target or an increased drug efflux from the cell (Chu 1994;el Deiry 1997;Grude et al. 1998;Harrison 1995;Lehne 2000;Lowndes & Murguia 2000;Ringborg & Platz 1996;Sancar 1995;Smets 1994). Most of these mechanisms impinge upon the interaction of a drug with its cellular target or immediate consequences of such an interaction. For example, a decrease in the cellular levels of topoisomerase II thwarts the efficacy of certain topoisomerase II inhibitors, and enhanced levels of glutathione increase resistance to DNA alkylating agents (Volm 1998b). Independent of the mechanism the cancer cell posseses, the end point is the same namely chemoresistance or radioresistance. Apoptosis resistance as a category of drug resistance is believed to be the underlying mechanism responsible for a significant number of cases that fail to respond to cytotoxic therapy (Dive 1997b).

4.2 Aim

Cholangiocarcinoma cells express the antiapoptotic proteins Mcl-1 and Bcl-X_L. Their role in the resistance of cholangiocarcinoma to chemotherapy and radiotherapy in unknown. To determine whether the Mcl-1 and Bcl-X_L expressing human cholangiocarcinoma cell lines Tfk-1 and Egi-1 are resistant to the apoptosis, experiments using chemotherapy or radiotherapy were performed *in vitro* with the generation of dose response graphs (curves).

4.3 Materials and Methods

Human cholangiocarcinoma cell lines Tfk-1 and Egi-1, which are both p53 and P-Glycoprotein negative and colorectal cell line HT29 were maintained in culture as described in chapter 2. Experiments were performed *in vitro* over a 96 hour period. Monitoring the loss of the mitochonadrial membrane potential $\Delta\Psi m$ at the single cell level (chapter 2) was used to measure the kinetics of apoptosis. The chemotherapy agents used were the antimetabolite 5FU and topoisomerase II inhibitor etoposide (VP16), along with UV and X-ray irradiation. In addition, diamide (5N,N-dimethylamide) was used as a directly acting mitochondrial apoptosis inducing agent (Halestrap, Woodfield, & Connern 1997). This drug alters the conformation of the mitochondrial permeability transition pore complex (PTPC) by oxidising critcal vicinal thiols within the compnent adenonucleotide translocator (ANT) making it a good inducer mitochondrial permeability transition (MPT), cytochrome c release and apoptosis (Huang, Pringle, & Sanadi 1985;Zamzami et al. 1998).

Cell lines studied in this chapter included the human cholangiocarcinoma cell lines Tfk-1, Egi-1 and the colorectal cell line HT29. The response of the HT29 cell line to chemotherapy has been studied previously and it is known to be resistant to apoptosis induction *in vitro* (Cesaro et al. 2001;Roy et al. 2001;Suzuki, Tomida, & Tsuruo 1998;Wright et al. 1999), unlike Tfk-1 and Egi-1 that have not been previously studied. This allowed for comparisons between the responses of HT29 and those of Tfk-1 and Egi-1

4.3.1 *In vitro* culture conditions and apoptosis stimulation

Egi-1 and Tfk-1 cells from exponential growing cultures were harvested by trypsinization. 2 x 10^5 cells were then re-suspended in culture medium before being plated onto 24 well flat bottomed plates (Gibco). The cells were allowed to adhere to the bottom of the plate overnight.

Chemotherapy

The doses of the chemotherapy drugs 5FU, Gemcitabine and Vp16 were between 1nM to 100uM (6-log concentration). Cells growing in 24 well flat-bottomed plates in culture were incubated with each dose of the chemotherapy drug in triplicate. Experiments were terminated at regular 24 hour intervals and the treated cells collected for either apoptosis measurement or cell cycle analysis.

UV and X-Ray Irradiation

Experiments involving UV irradiation was performed by exposing the cells in culture for either 1, 3 or 5 minutes to UV irradiation emitted from 120mJ/cm2, Chromato-UV trans-illuminator, (model TM-20n). X-Ray irradiation treatment of the cells at a dose between 50-1000cGy came from a 6MV linear accelerator (Elekta oncology SL75/5) using the protocol outlined in chapter 2.

Mitochondrial Oxidation

The mitochondrial targeting divalent thiol-reactive agent diamide, which causes thiol cross-linking is capable of altering the conformation of the mitochondrial mega-channel. Doses used ranged from 100-500μM to treat the cells, each experiment was performed in triplicates.

4.3.2 Cell cycle Studies

The cell cycle response of the cholangiocarcinoma cell lines was monitored following treatment with DNA damaging therapies. This was performed not only to study the cycle changes that were occurring but also to measure apoptosis using the proportion of cells with subnormal levels of DNA (Sub-G1 population).

Half of the cells stimulated with chemotherapy were collected at the end of each experiment as outlined above. Cells were re-suspended in 100ul of phosphate buffered saline (PBS) and spun down to a pellet before being permeabilised by re-suspending in 70% ethanol on ice (4°C) for 30 minutes. Cells were then washed three times in PBS and resuspended in 500ul of PBS containing 50ug/ml propidium iodide (PI) (Sigma,

Dorset, UK) and RNAse A (Sigma, Dorset, UK) at 200ug/ml for 30 minutes at room temperature. Cell cycle analysis was performed as outlined in chapter 2.

Apoptosis Measurement

Apoptosis was detected and measured at single cell levels by flow cytometry using the multi-parametric technique as described in chapter 2, pages 99 to103.

Controls

Contol groups were unstimulated cells cultured along side stimulated cells.

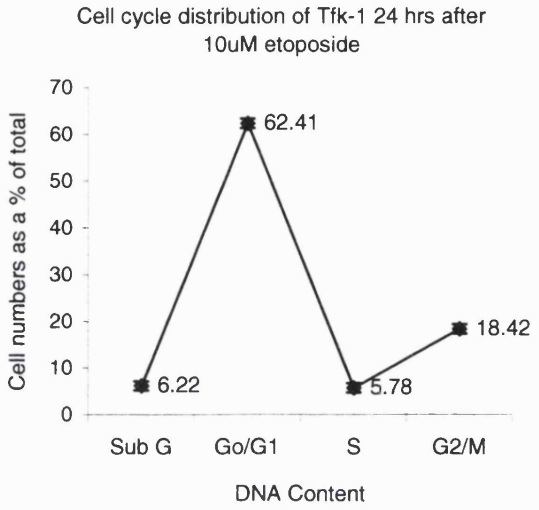
Statistics: All experiments were performed in triplicates, results are expressed as mean ± standard error. Comparison of differences employed unpaired two-tailed Student's t-test with a significance level of 0.05.

4.4 Results

In vitro exposure of human cholangiocarcinoma cells to the DNA damaging effects of either chemotherapy or radiotherapy causes the cells to arrest/pause at G_2/M checkpoint in the cells cycle (figure 4a and b). The longer the experiment time the greater the fraction of arrested cells (figure 4a, b and i). This effect was noticed to be independent of the drug dose.

Analysis of the quantity of such cells undergoing mitochondrial membrane permeability transition and apoptosis showed that over the 48-96 hour sampling time for the experiments only a small proportion of the total cell numbers were undergoing apoptosis (figure 4c, d, e and f). This response differed only marginally with changes to the DNA damaging agent employed and the sampling time. The low proportion of cholangiocarcinoma cells undergoing mitochondrial depolarisation and apoptosis was compared to the Bcl-2 expressing lymphoma cells line SUDHL4 and the colonic carcinoma cell lines HT29. By comparison it can be seen that irresepective of the apoptosis inducing method used both SUDHL4 and HT29 cells were at least twice more likely to undergo apoptosis than Tfk-1 or Egi-1 (figure 4g and 4h). The sub-G1 population detected when analysing the DNA content for cell cycle studies (another recognised method of quantifying apoptosis) was found to correspond well with the proportion of cells undergoing mitochondrial permeability transition (MPT)(figure 4i).

Interestingly, the response following treatment of Tfk-1 and/or Egi-1 cells with diamide differed significantly from the response to chemo-radiotherapy with the cells readily undergoing apoptosis in a dose dependent manner after 24 hours (figure 4j).



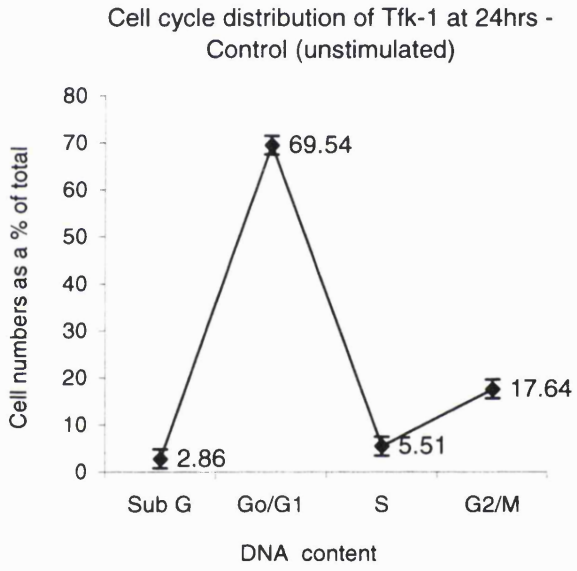
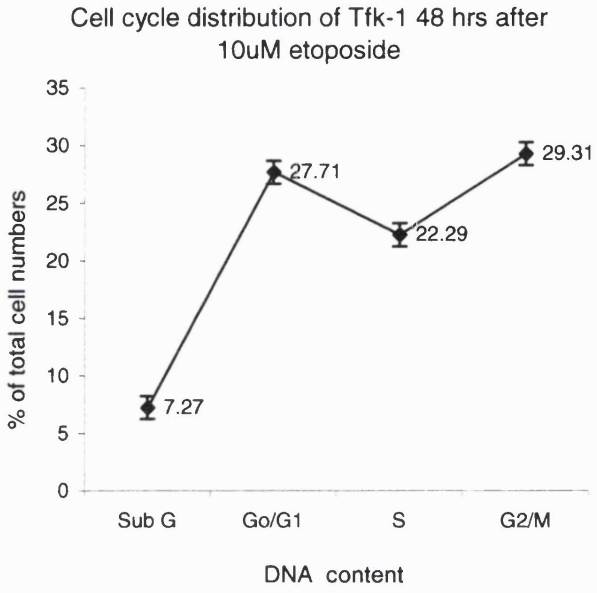


Figure 4 a

Graphs showing the cell cycle distribution of Tfk-1 cells 24 hours after exposure to 10μm VP16 along with control – unstimulated (*Go/G1- Growth phase, S-synthetic phase, G2/M- Mitotic phase, sub-G fragmented DNA –apoptosis)*



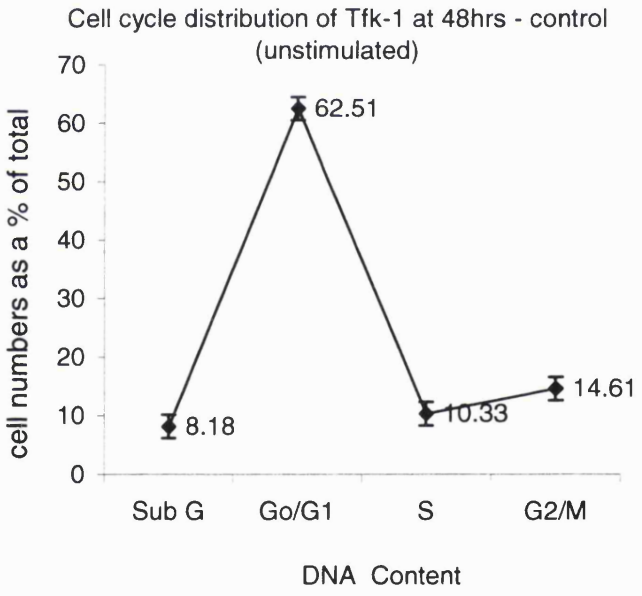


Figure 4 b

Graphs showing the cell cycle distribution of Tfk-1 cells 48 hours after exposure to 10μm VP16 along with control – unstimulated.

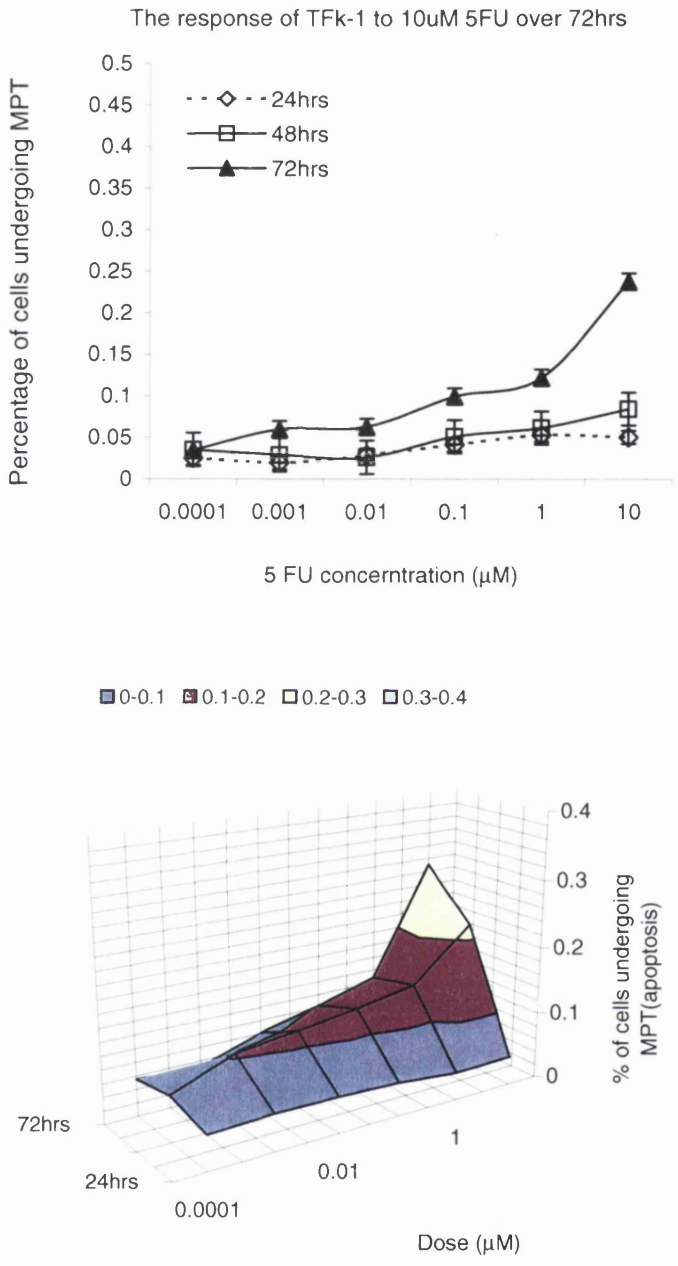
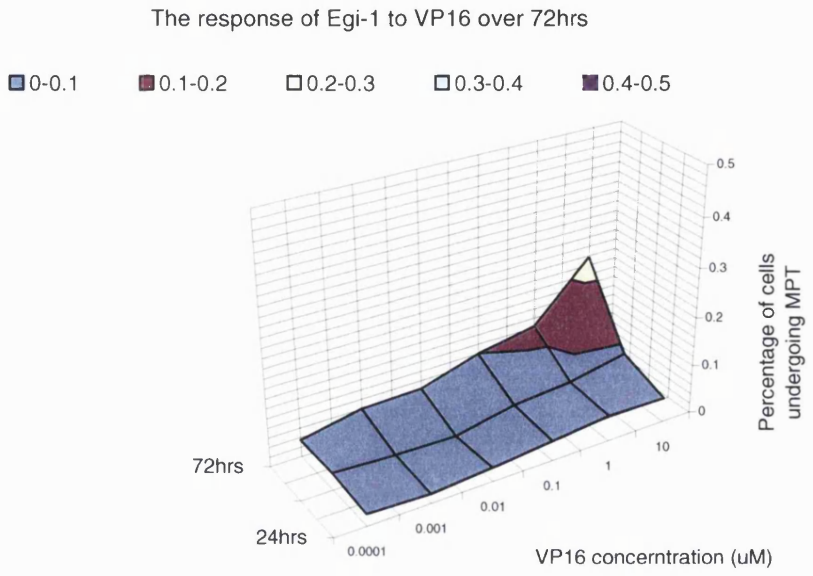


Figure 4c

The dose response profile of Tfk-1 to a 6-log concentration of 5 FU over 72 hours presented as both linear and surface graphs



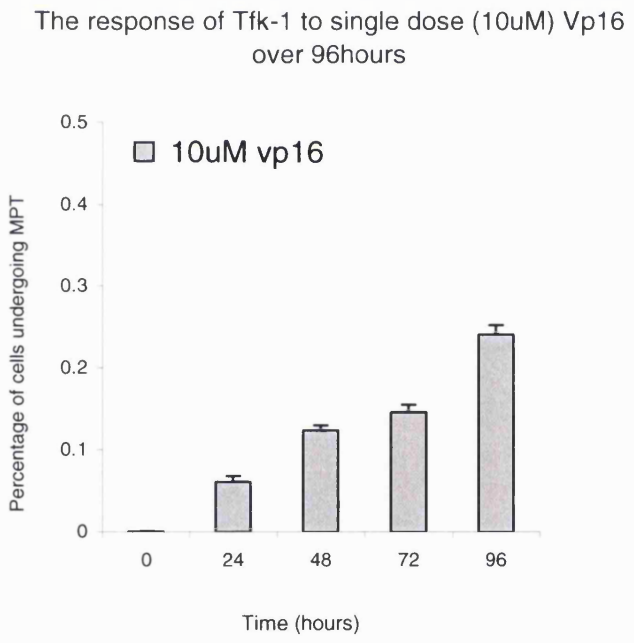
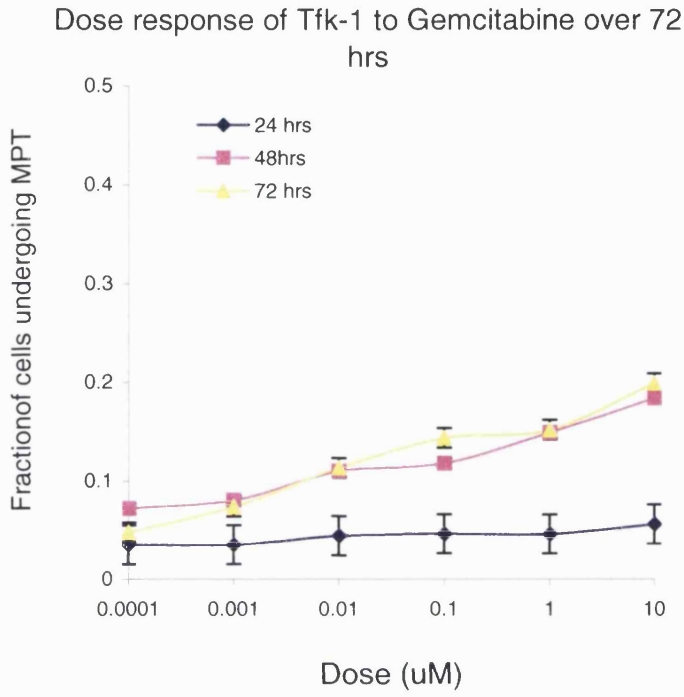


Figure 4d

Surface graph and histogram showing the response of Egi-1 and Tfk-1 to dose range and single dose of Vp16 over 72 and 96 hours respectively



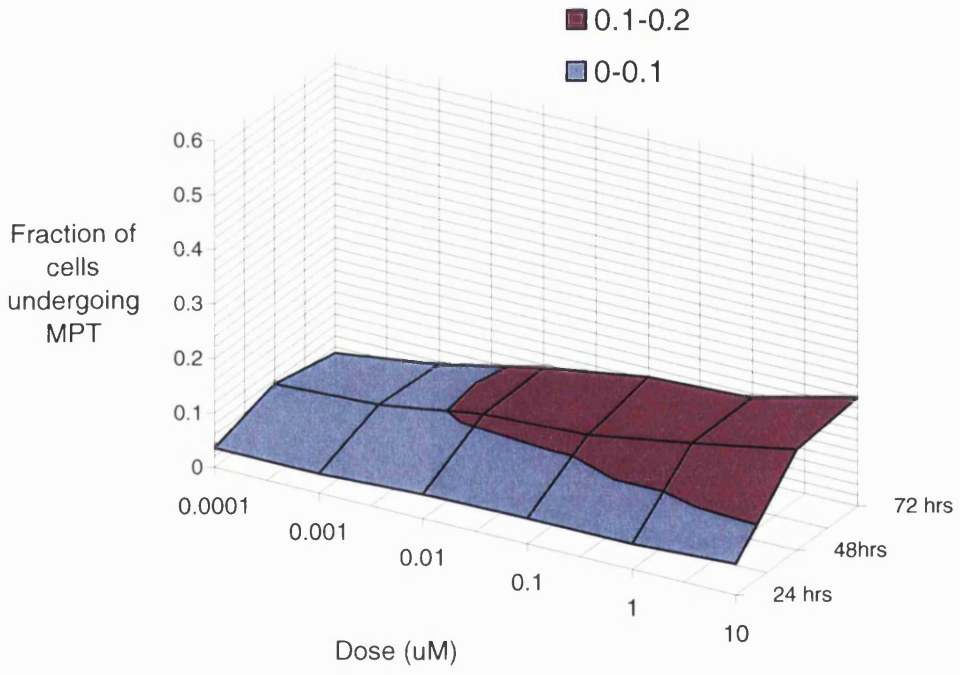


Figure 4e

The changes in the apoptosis response of Tfk-1 cells over 72 hours to Gemcitabine

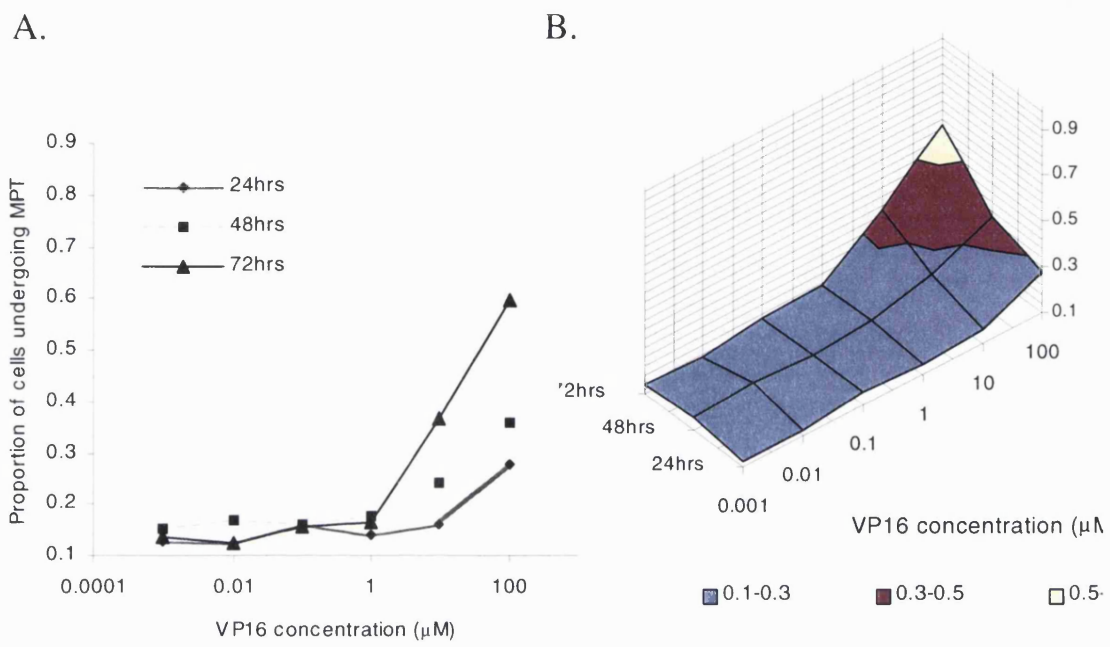
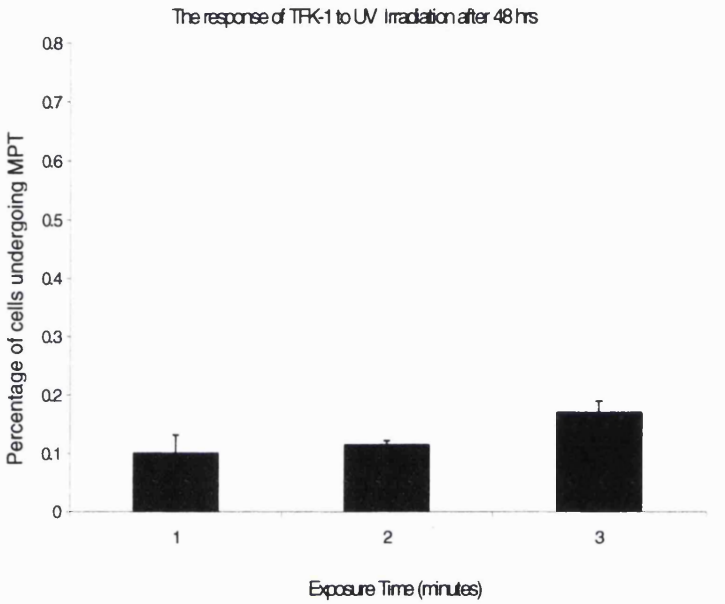


Figure 4F Graphs illustrating the response of SUDHL4 cells 72 hours after treatment with VP16 shown as a histogram and a surface gragh



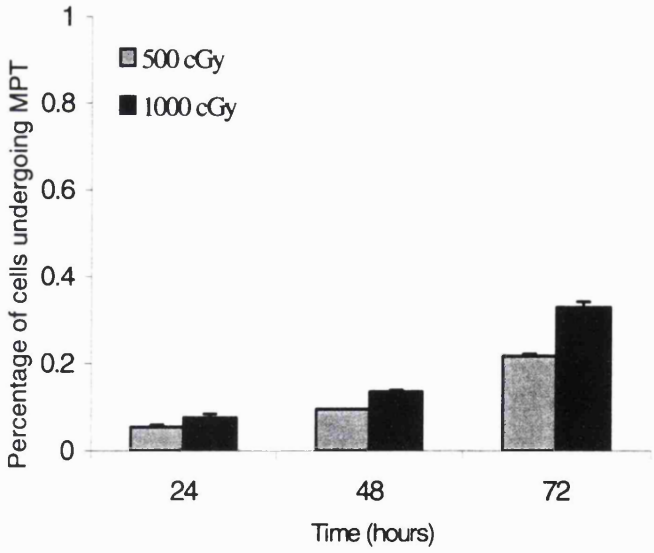


Figure 4g

Histograms demonstrating the response of Tfk-1 to both UV and X-ray Irradiation over 72 hours

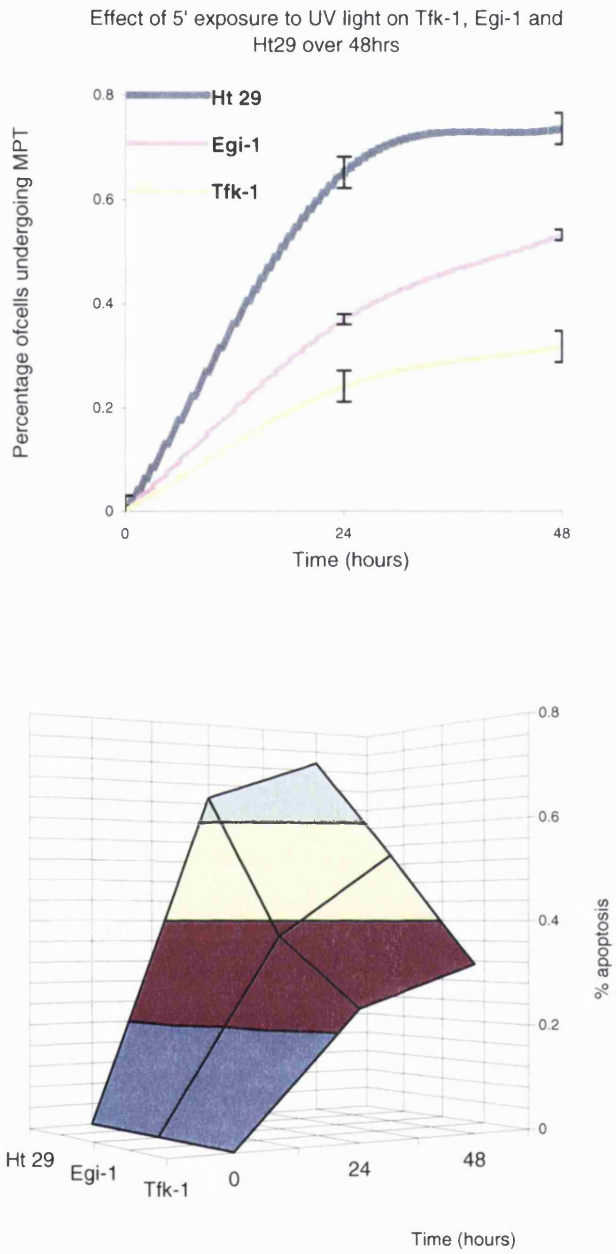
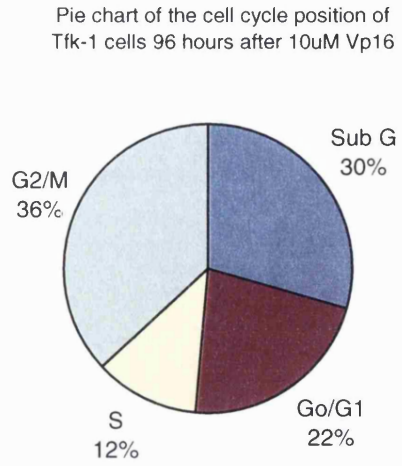


Figure 4h

Graphs comparing the different responses of Tfk-1, Egi-1 and HT29 cell lines to a 5' exposure to UV irradiation over 4 hours



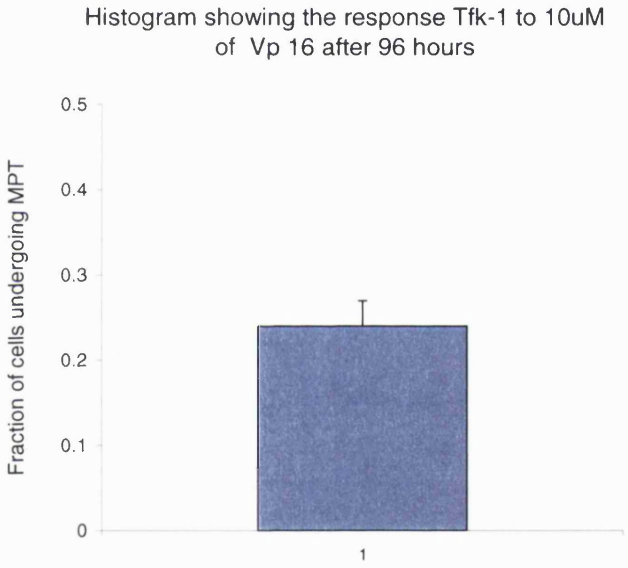


Figure 4i

The fraction of total live cell population in the Sub-G group (blue sector of pie chart) corresponds to the proportion of cells found to be undergoing membrane permeability transition 96 hours after treating Tfk-1 with 10uM Vp16 (Histogram).

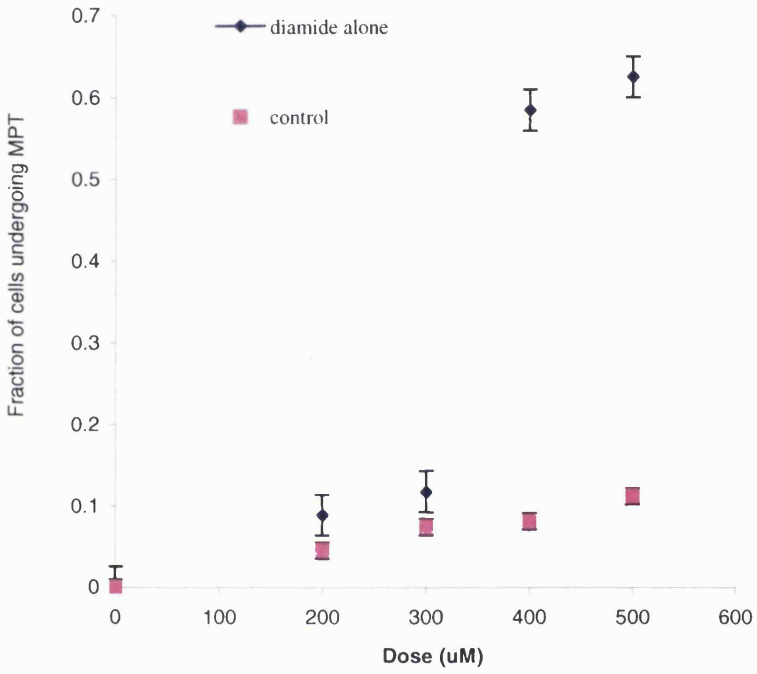


Figure 4j

The response of Tfk-1 to 4 different doses of the mitochondrial mega channel cross-linking agent diamide after 24 hours.

4.5 Discussion

The apoptotic response of human cholangiocarcinoma cells *in vitro* to cytotoxic (DNA damage) has not previously been reported. The present study demonstrates that the majority of human cholangiocarcinoma cells 96 hours after exposure to a range of DNA damaging agents fail to undergo apoptosis *in vitro*. When these responses are compared with to other cell types it can be seen that the cholangiocarcinoma cells are found to be more resistant to apoptosis induction. It can be seen that Egi-1 was marginally more susceptible than Tfk-1 to apoptosis a feature that was independent of the method of DNA damage. The factor(s) responsible for this difference are for now remain unknown.

The cell cycle analysis performed simultaneously on treated cells confirm that following chemotherapy the cells undergo G2/M arrest. This was found to occur even when the cells were exposed to low drug doses. Despite an increasing number of cells arrested at G2/M in the cycle over time, the number of cells undergoing mitochondrial permeability transition and apoptosis remained low. Whether these cells are capable of repairing their DNA and re-entering the cycle or eventually die is unknown (Lowndes & Murguia 2000;Sancar 1995).

When the human cholangiocarcionoma cells were incubated with increasing doses of diamide, a drug that directly targets the mitochondria bypassing the nucleus, the magnitude and rate of apoptosis of the human cholangiocarcinoma cells increased. The significance of this observation lies in the fact that it confirms that when adequately stimulated, in this case by this mitochondrial toxin, cholangiocarcinoma cells readily undergo apoptosis and this shows that cholangiocarcinoma cells possess an intact and functional post-mitochondrial pathway to apoptosis. Comparing the

differences in the response of the human cholangiocarcinoma cells to diamide with that of DNA damage suggests that defect in the apoptosis pathway within the cholangiocarcinoma cells may be located at some point between the generation of nuclear signals, their connection and interpretation at the mitochondrial level (Green & Reed 1998b).

These results demonstrate that cholangiocarcinoma cells are resistant *in vitro* to apoptosis induced by DNA damage. This response is in keeping with the hypothesis that the high expression of Bcl-X_L and Mcl-1, members of Bcl-2 antiapoptotic protein family by cholangiocarcinoma cells may be playing a part in attenuating the apoptotic response in cholangiocarcinoma cells. The experiments in the following chapter set out to investigate whether a link exists between the mitochondrial located antiapoptotic proteins in cholangiocarcinoma cells and their resistance to apoptosis.

Chapter 5

Mitochondrial Targeted Modulation of Cholangiocarcinoma Apoptosis *in vitro and in vivo*

5.1 Introduction

Over the last decade there has been enormous progress in the understanding of apoptosis. Chromatin condensation and DNA fragmentation are well characterised cellular events that occur in apoptosis. For a long time researchers were unclear as to whether other targets besides the nucleus were affected during apoptosis. Recently, the mitochondria have been closely linked to the process of apoptosis. Emerging evidence supports their role as central players in apoptosis, for example, in a cell-free system only mitochondria enriched cytoplasm fractions are capable of inducing nuclear apoptosis (Newmeyer, Farschon, & Reed 1994). Reactive oxygen species (ROS) may participate as effector molecules (Sandstrom, Mannie, & Buttke 1994). Although the source of the ROS has not been characterised, it is known that the ubiquitous complex of the respiratory chain is a major source of superoxide free radicals. Disruption in mitochondrial function as indicated by changes in the mitochondrial transmembrane potential which ($\Delta\Psi m$) precedes nuclear changes in apoptosis (Vayssiere et al. 1994) (Zamzami et al. 1996b) permitting the measurement of changes in $\Delta\Psi m$ as a nonnuclear parameter for detecting apoptosis. A multi-protein complex forms a large channel, the mitochondrial permeability transition pore complex (PTPC), which spans the outer and inner mitochondrial membranes at contact points. The PTPC functions as a

Ca^{2+}, voltage, pH, redox gated channel, with several levels of conductance (Bernardi & Petronilli 1996;Ichas, Jouaville, & Mazat 1997). Opening of this channel, known as mitochondrial permeability transition (MPT), results in the increased permeability of the mitochondria to solutes with molecular masses < 1500 daltons, resulting in the uncoupling of respiration with collapse of $\Delta\Psi m$, the cessation of ATP synthesis and defects in the ADP import into the mitochondrial matrix from the cytosol. As the F_1F_0 ATPase uses ADP as a substrate, the F_1F_0 ATPase fails to function without matrix ADP, since the return of protons (H+) from the intermembrane space to the matrix through the ATPase is coupled to ATP synthesis H+ accumulates in the intermembrane space. This exerts an net osmotic effect resulting in the swelling of the intermembrane space and eventual rupture of the less compliant outer mitochondrial membrane with the release of apoptogenic proteins (caspase activators) such as inter-membrane cytochrome c and apoptosis inducing protein (AIP) (Kluck et al. 1997;Susin et al. 1996a;Vander Heiden et al. 1997).

The exact composition of PTPC is unknown, however, it is thought to involve proteins from the cytosol (Hexokinase) (Nakashima et al. 1986), the outer mitochondrial membrane (voltage dependent anionic channel -VDAC and mBzR)(Colombini 1980;McEnery 1992) (Petronilli et al. 1187), the internal membrane (adenine nucleotide translocator -ANT)(Brustovetsky & Klingenberg 1996) and the matrix (cyclophilin D). As a consequence the permeability transition pore contains multiple targets for endogenous regulators. In intact cells and isolated mitochondria, several pro-apoptotic second messengers induce PT pore opening: Ca2+, pro-oxidants, nitric oxide, ceramide and caspase 1. Members of

the Bcl-2 family of apoptosis regulating proteins are located preferentially in the outer mitochondrial membrane, Bcl-2 and Bcl-X_L proteins have been shown to interact directly with the PTPC and prevent MPT (Marzo et al. 1998;Marzo, Brenner, & Kroemer 1998;Vander Heiden et al. 1997;Vander Heiden et al. 1999c), while Bax promotes MPT (Shimizu, Narita, & Tsujimoto 1999). Similarly, adenine nucleotide translocator (ANT) Ligands such as bongkrekic acid and atractyloside reduce and enhance the probability of PT, respectively (Halestrap & Davidson 1990;Marty et al. 1992). Experiments using purified mitochondria derived from Bcl-2 transfected cells show that in the presence of Bcl-2 atractyloside failed to induce apoptosis (Zamzami et al. 1996b). The three dimensional structure of Bcl-X_L has been found to be closely related to the pore forming domains of bacterial toxin such as diphtheria toxin and colicins (Muchmore et al. 1996). In a manner that is analogous to these bacterial toxins, Bcl-X_L can form functional ionic channels in synthetic lipid membranes. These channels have multiple conductance states, are cationic selective and insert in a pH sensitive manner (Minn, Velez, Schendel, Liang, Muchmore, Fesik, Fill, & Thompson 1997). The potential for Bcl-X_L to form channels in membranes suggest that part of its function may include the regulation of membrane permeability of the intracellular organelles to which it localises. Such data, in combination with the findings from experiments involving reconstituted PTPC in liposomes supports a role for Bcl-2 and Bcl-X_L as endogenous inhibitors of MPT and resulting apoptosis. Overall, physiological modulators of MPT (Ca2+, Mg2+, ADP, ATP, ceramide, glutathione) and Bcl-2 homologues together determine the fate of the cell.

The PTPC can be regulated directly or indirectly inorder to overcome the antiapoptotic effects of the Bcl-2 family, for instance Bcl-2 expression can be blocked by using antisense oligonucleotides both *in vitro* and *in vivo,* this is associated with a reduction in the apoptosis threshold (Hatfield et al. 1982;Koty, Zhang, & Levitt 1999;Tompkins et al. 1990;Viranuvatti et al. 1972). Using pharmacololgical agents that are specific pore agonist or antagonists it possible to enhance the cellular apoptotic response. Pk11195 is an example. This isoquinolone carboxyamide compound is a ligand of the mitochondrial benzodiazepine receptor (mBzR) that is an effective apoptosis sensitiser in the presence of diverse apoptosis stimuli including rotenone (Pastorino et al. 1041), tumour necrosis factor α (Pastorino et al. 1996), etoposide, dexamethasone, ceramide, UV light (Hirsch et al. 1998c). Furthermore, Pk11195 induces the release of Apoptogenic factors from isolated mitochondria and collapse of the mitochondrial membrane potential, leading to apoptosis in malignant cells in the presence of high Bcl-2 expression (Hirsch et al. 1998c). Pk11195 reduces apoptosis threshold at concentrations that do not exhibit cytotoxic efficacy per se. In vivo, diazepam (which binds the mBzR as well as the central benzodiazepine receptor with nanomolar affinity) has been shown to synergise with lonidamine in its anti-glioma activity (Miccoli et al. 1998) (Ravagnan et al. 1999). Other agents include arsenic (Larochette et al. 1999) and diamide (Zamzami et al. 1998). A number of such agent have had success in the clinical setting, arsenic trioxide has been found to have significant therapeutic efficacy against promyelocytic leukaemia (Soignet et al. 1998). Protoporphyrin IX (PPIX) is an endogenous ligand with nanomolar affinity for the mBzR.(Snyder, Verma, & Trifiletti

1987;Verma, Nye, & Snyder 1987;Verma & Snyder 1989). 5 δ aminolaevulinic acid is its precursor used as a pro-drug in photodynamic therapy (PDT) (Verma et al. 1998). It is yet to be proven exactly how PDT sensitises malignant cells, however it is thought to involve lowering the apoptosis threshold.(Pastorino, Simbula, Gilfor, Hoek, & Farber 1041).

5.2 Aim

In the preceeding chapters, Cholangiocarcinoma cells *in vitro* have been shown to be resistant to apoptosis (MPT) following DNA damage. This chapter set out to test the hypothesis that the failure to undergo apoptosis in cholangiocarcinoma cells may be wholly or partly due to dysfunction of the PTPC leading to a high threshold for MPT.

5.3 Material and Methods

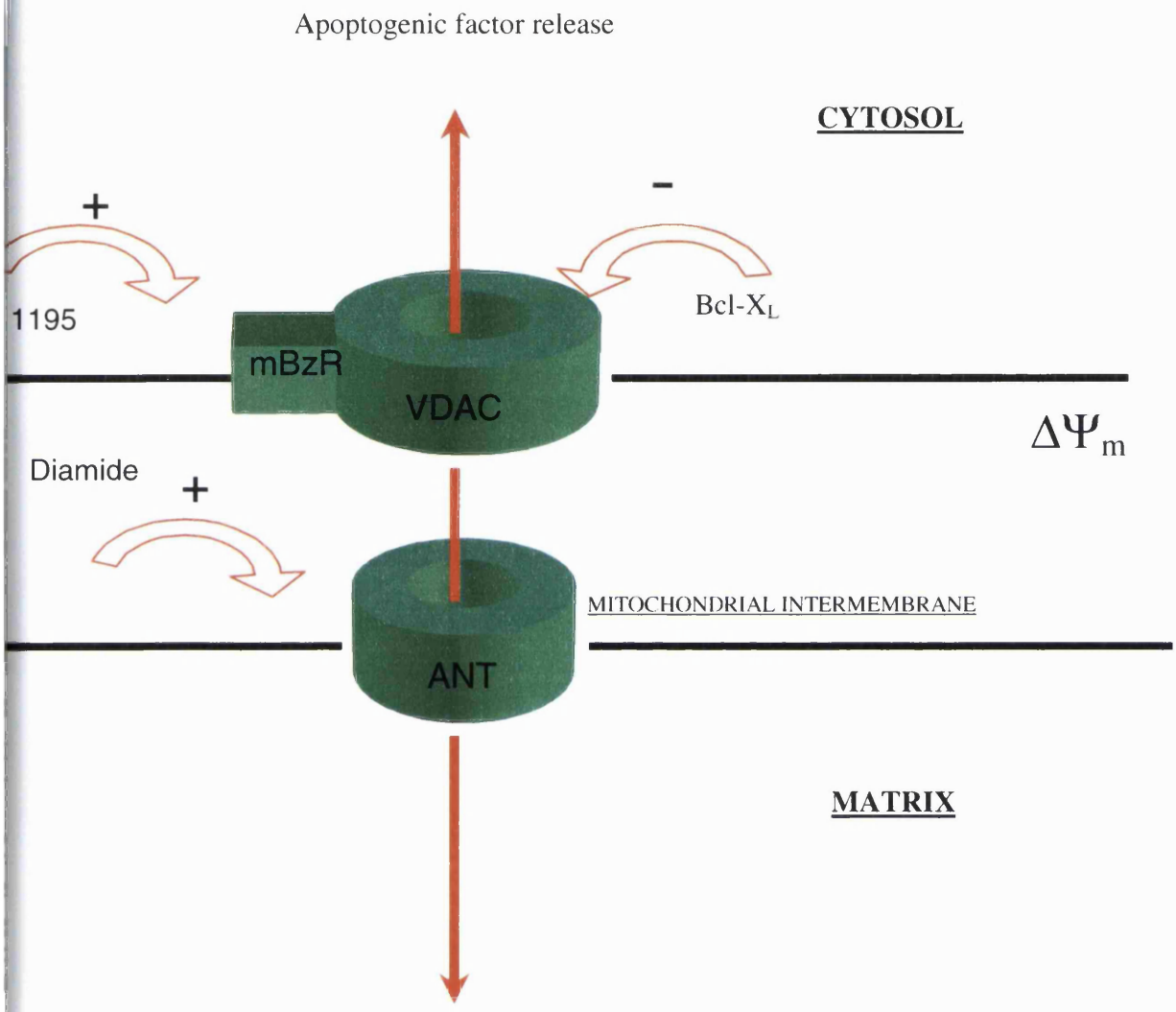
Bcl-X_L and Mcl-1 expressing human cholangiocarcinoma cells lines Egi-1 and Tfk-1 were treated in culture with chemotherapy, UV and X-ray irradiation with or without the addition of PTPC modulation. Cellular apoptosis was measured as previously described in chapter 2. The cell lines were then implanted as subcutaneous xenografts on the back of SCID-NOD mice. Xenograft growth was monitored before and following the treatment with etoposide alone or in combination with Pk11195.

5.3.1 PTPC Modulating Agents

There is a growing number of pharmacological agents/molecules available that have specific actions on the mitochondrial PTPC. Their use in the field of apoptosis research is invaluable, not only as tools that can be adapted for use in identifying relevant apoptosis pathways, but also have potential as new therapeutic agents.

Pk11195

This isoquinolone carboxyamide derivative is a ligand of mBzR. It has a high affinity for its receptor and binds at nanomolar concentrations (Kozikowski et al. 1997). Pk11195 has been shown to reverse *in vitro* Bcl-2 protein mediated cytoprotection in cells that over express Bcl-2 (Hirsch et al. 1998c). The mBzR is expressed by a variety of cell types including biliary cells and hepatocytes (figure 5a).



MITOCHONDRIAL PERMEABILITY TRANSITION (MPT)

Figure 5a

PK11195 and Diamide antagonising anti-apoptotic proteins block MPT by inhibiting the opening of the mitochondrial permeability transition pore

Diamide (5 N, N-dimethylamide)

This thiol crosslinking agent directly interferes with the function of PTPC by oxidising critical vicinal thiols hence encouraging MPT (Huang, Pringle, & Sanadi 1985;Marchetti et al.1997;Petronilli et al. 1994b;Publicover et al. 1979b;Publicover et al. 1979a;Zamzami et al 1998). Although the precise mode of action remains unclear at present, data gathered from experiments using whole cells, isolated mitochondria and purified ANT extracts, suggest that diamide has a direct effect on the ANT component of PTPC that leads to MPT (Costantini et al. 2000)(Figure 5a).

5.3.2 Apoptosis Induction by DNA damage therapies

Apoptosis stimulation involved dose response studies according to the protocols for chemotherapy and irradiation induced DNA damage outlined in chapter 2.

5.3.3 Modulation of Cholangiocarcinoma Apoptosis using PTPC Targeting Agents

The attenuation of apoptosis kinetics in cholangiocarcinoma cells by PTPC modifying agents was conducted *in vitro*. Experiments were performed over a maximum time frame of 96 hours. The principles of the experimental design focuses on observing the changes in apoptotic response of cells that may occur in the presence or absence (controls) of a PTPC modulator. All experiments were in triplcates and reproduced on more than two separate occasions. The protocols are outlined below:

Detection of Benzodiazepine receptor (mBzR) expression: Whether the mBzR was present in the human cholangiocarcinoma cell Tfk-1 and Egi-1 was investigated using a specific mBzR fluorescent probe 7-nitro-2, 1, 3-benzoxadiazol-4-yl derivative (NBD FGIN-1-27 analogue) (Kozikowski et al.1997) and the mitochondrial flourochrome chloromethyl-X-rosomine (CMXRos). Human cholangiocarcinoma cells suspended in culture medium were incubated for 45 minutes with 1μM NBD FGIN-1-27 and chloromethyl-X-rosomine (CMXRos) at 37°C. After being mounted on slides, images were captured using a Zeiss Axioskop fluorescence microscope running Iplab spectrum image analysis software (version 3.1.1).

Activity and Tumour bioavailability of mBzR ligands *in vivo*

Pk11195 was administered daily for 3 consecutive days at 10mg/kg by intraperitoneal (i.p) injections to SCID-NOD mice bearing Tfk-1 and Egi-1 xenografts. This dose has been shown to exhibit *in vivo* activity in humans by attenuating lorazepam discontinuation effects (Byrnes et al. 1993). VP16 was administered i.p at a therapeutic dose of 100mg m^{-2} on the first day of treatment (Whelton et al 1969).

To investigate the tumour bioavailability of Pk11195, mice were administered i.p NBD FGIN-1-27 at 10mg/kg/day providing an identical schedule as for Pk11195 treated groups 1 hour before they were sacrificed. Fluorescence microscopy of the slides containing cells mechanically disaggregated from xenografts was as previously described.

Chemotherapy induced apoptosis *in vitro:* 75cm^2 flasks containing exponentially growing cells that were approximately 60-70% confluent were split using trypsin/EDTA

(x 1). 1x 10^4 cells were then re-cultured in 12 well flat-bottomed plates, allowed to adhere over night and treated the following day with either 10μM 5 Fluorouracil or 10μM Vp16, with or without 75μM Pk11195.

Ultraviolet and X-ray Irradiation induced apoptosis *in vitro:* 12 well flat-bottomed plates containing growing Tfk-1 or Egl-1 cells in culture were exposed either to ultraviolet (UV) irradiation (120mJ/cm^2, Chromato-UV-E trans-illuminator, model TM-20) for 5 minutes or X-ray irradiation at a dose of 0.5-1Gy from a 6MV linear accelerator (Elekta oncology SL75/5) again with or without Pk11195 at a dose of 75μM.

Measurment of mitochondrial dysfunction by flow cytometry: Mitochondrial dysfunction, one of the parameters for detecting apoptosis was measured at single cell level by detecting the collapse of the inner mitochondrial membrane potential, $\Delta\Psi_m$ as described in chapter 2 (Cai, Yang, & Jones 1998;Hirsch et al.1998b;Marchetti et al. 1995). Multi-parametric cell analysis was performed at single cell resolution by flow cytometry running the Cellquest software (versio*n* 3.2.1). When sampling time was reached the experiment was terminated and all floating and adherent cells (after trysinisation) from each well collected in labeled FACS tubes, washed and spun down to a pellet, then re-suspended in culture medium ready for staining.

Phosphotidylserine expression: This caspase dependent event was measured using the annexin V assay. Cells in suspension were incubated in calcium buffer and 5μL FITC Annexin V for 15 minutes in the dark. Cells were washed twice in Hank's buffered saline solution and then re-suspended in 20μg/ml propidium iodide for 10 minutes before analysis by Flow cytometry.

Pk11195 anti-tumour activity in immunodeficient mice-xenograft models: Severe combined immunodeficient non-obese diabetic (SCID-NOD) mice were allocated for interscapular subcutaneous inoculation of 5 x 10^7 Egi-1 or Tfk-1 cells in 1mL of medium. Tumors grew in all cases and usually became palpable within 2-3 weeks. Using calipers tumor size was measured on alternate days along three different planes (diameters) and growth monitored for 1 week before the onset of treatment i.e when normalized mean diameter of tumor xenograft was 120-150% of its original size (1^{st} time measured, Day 1 on graph figure 5g), allowing for within-xenograft comparison of pre-treatment and post treatment growth rates. Mice were randomly subdivided into three treatment groups per cell line xenograft (85μg/mouse/day of VP16, 85μg/mouse/day of VP16 + 10mg/kg/day of PK11195 and saline (control)) making a total of 27 mice per cell line per single experiment (triplicates). Mice were treated daily for three days on two separate occasions 16 days apart by the daily intraperitoneal administration of either VP16+/-Pk11195 or saline. Tumor growth (T_t) during the experiment was calculated using the mean of three tumour diameters (d_1 to d_3) at time t, and normalising against the initial tumour size measured at time t = 0 (T_0), i.e.

$$T_t = \frac{\left[\frac{\sum_{i=1}^{3} d_i}{3}\right]_t}{T_0}$$

Statistics: Results are expressed as mean ± standard error. Comparison of differences employed unpaired two-tailed Student's t-test with a significance level of 0.05.

5.4 Results

The human cholangiocarcima cell lines Tfk-1 and Egi-1 express the antiapoptotic proteins Mcl-1 and Bcl-X_L, just like human cholangiocarcinoma cells from resected tumours. These two cell lines are resistant to chemotherapy and radiotherapy induced apoptosis in vitro for unknown reasons.

In the persecnce of Pk11195, the mBzR and Bcl-2 antagonist the response of both Tfk-1 and Egi-1 human cholangiocarcinoma cells was altered following treatement using chemotherapy or radiotherapy (Figures 5b and 5c respectively) as shown by the diffences in the kinetics of $\Delta\Psi m$ dissipation and apoptosis. For instance, the 28% fraction of apoptosis of Egi-1 cells at 72 hours following 1Gy X-ray irradiation became 92% following the addition of Pk11195 (Figure 5c), Tfk-1 cell apoptosis at 48hours increased from 26% to 53% following exposure to UV-irradiation (Figure 5b) in the presence of 75μM Pk11195. The magnitude of the observed 75μM Pk11195 effect was dependent both on the dose of the apoptosis stimulator used and the time the experiment was sampled after stimulation (figure 5c). This is consistent with an apoptosis sensitisation of both Egi-1 and Tfk-1 cells. In order to verify that the mitochondrial events measured resulted in caspase activation the caspase dependent annexin V assay was carried out following treatment and demonstrated an identical dose and time dependent rise in phosphotidylserine expression confirming apoptosis (figure 5d). Incubation of either cell line with 75μM Pk11195 on its own did not produce any increase in the baseline apoptotic rate confirming that 75μM Pk11195 on its own possessed no intrinsic cytotoxic effects on either cell line over a 96 hours period (Figure 5e).

Using NBD-FGIN-1-27 analogue (Kozikowski, Kotoula, Ma, Boujrad, Tuckmantel, & Papadopoulos 1997), the flourescent equivalent of Pk11195, we demonstrated that the mitochondria of the cholangiocarcinoma cells Tfk-1 and Egi-1 concentrated the mBzR probe in an identical distrubution as the mitochondrial flourochrome chloromethyl-X-rosomine (CMXRos) confirming mitochondrial targeting (figure5f). The ability of the probe to reach the tumor cells of the xenografts (figure 5g) following intar-peritoneal injection confirms the bioavailability of Pk11195 using this method of administration.

The ability of Pk11195 to alter the response of cholangiocarcinoma cell apoptosis was also studied *in vivo*. Xenografts grown on mice treated with Vp16 alone on two separate occasions showed no response, with a growth pattern similar to the control mice (Figure 5h). However, mice simultaneously treated with Pk11195 in addition to VP16 showed both growth slow down and regression of xenografts (Figure 5h). This effect was reproduced when the mice were treated for a second time (Figure 5h). Similarly, Egi-1 xenografts exhibited a similar attenuation in growth over the 72 hours of treatment (Figure 5i).

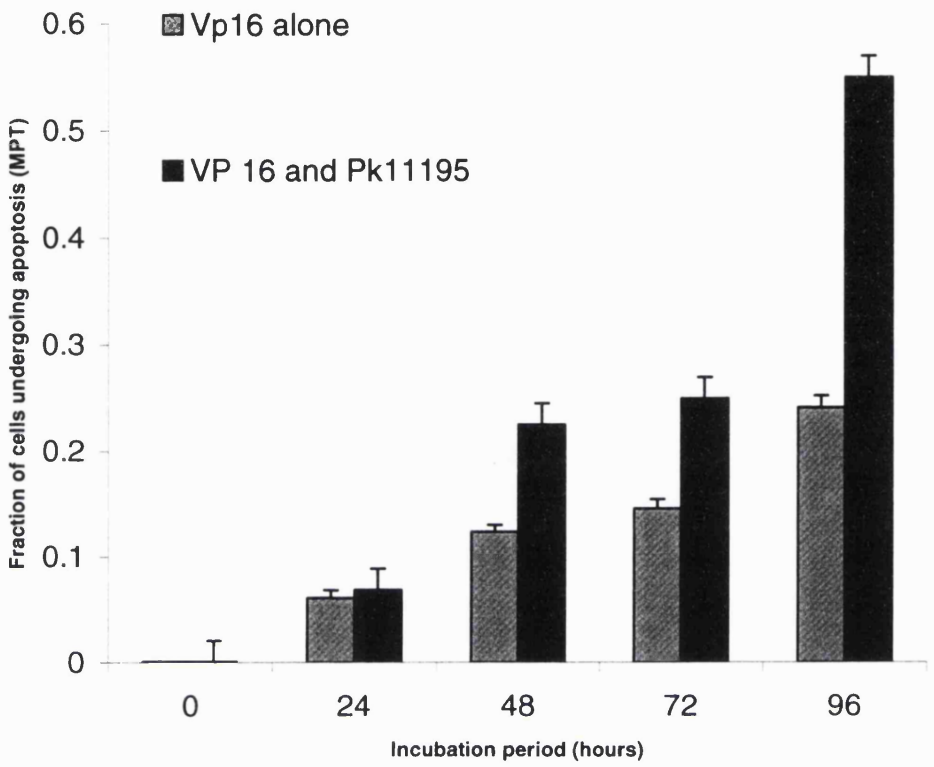


Figure 5b

The effects 10μM VP16 on the apoptosis rate of human cholangiocarcinoma cell line Tfk-1 over 96 hours when administered alone or in combination with Pk11195

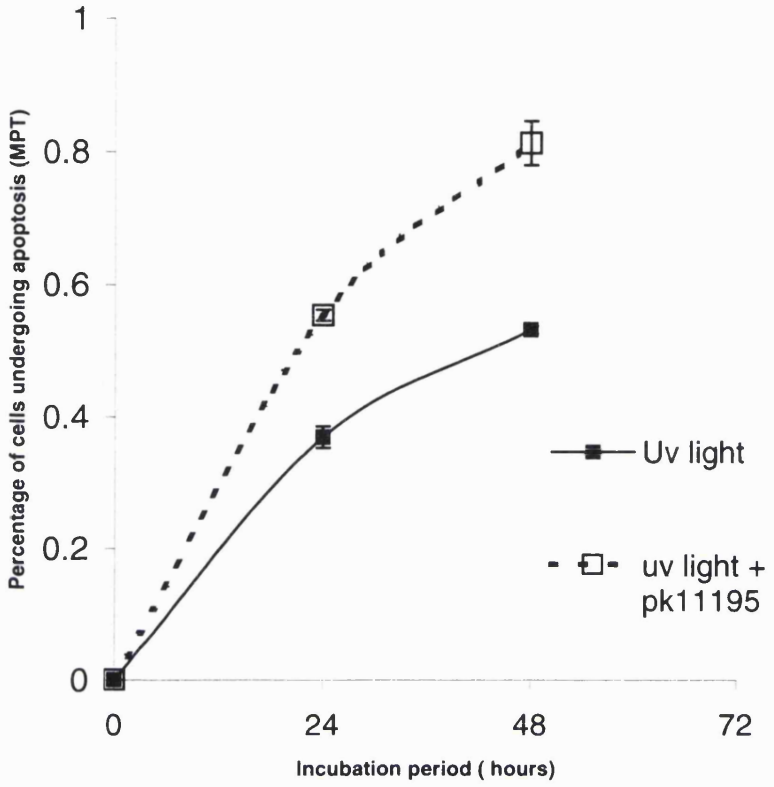


Figure 5c

The response of the human cholangiocarcinoma cell line Tfk-1 over 48 hours following a 5-minute exposure to UV irradiation with or without Pk11195

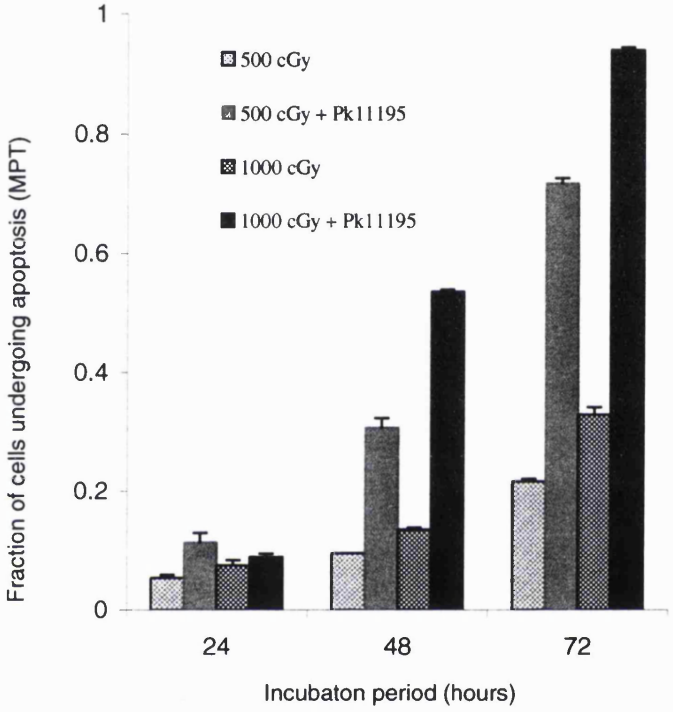


Figure 5d

Fraction of human Egi-1 cells undergoing apoptosis following treatment with 5 or 10 Gy X-ray Irradiation. Cells were sampled over 96 hours post stimulation with or without the addition of Pk11195

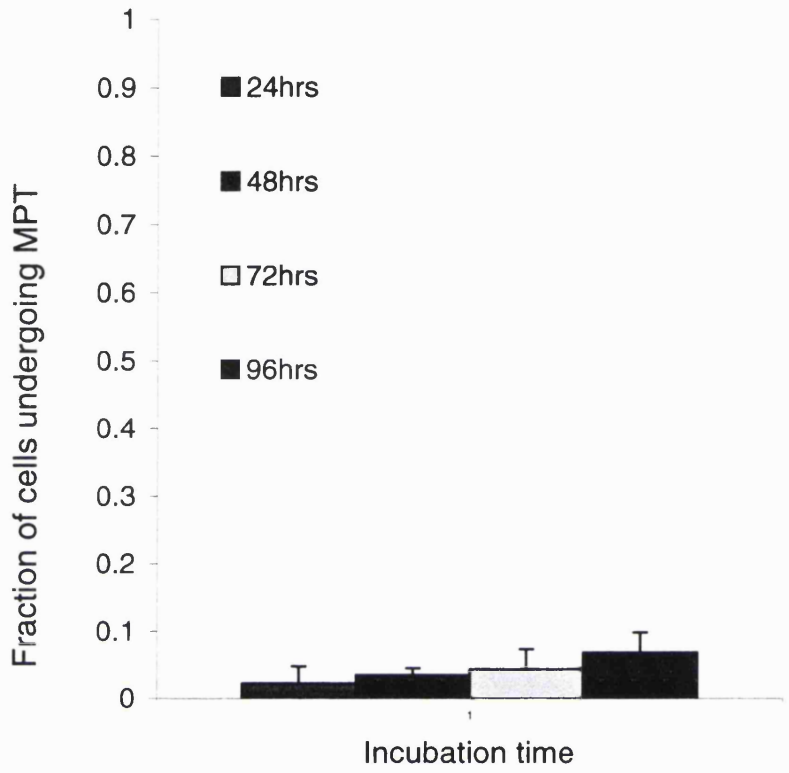


Figure 5e

The effect of 75 μM Pk11195 on its own when incubated with the human cells line Egi-1 cells over 96 hours

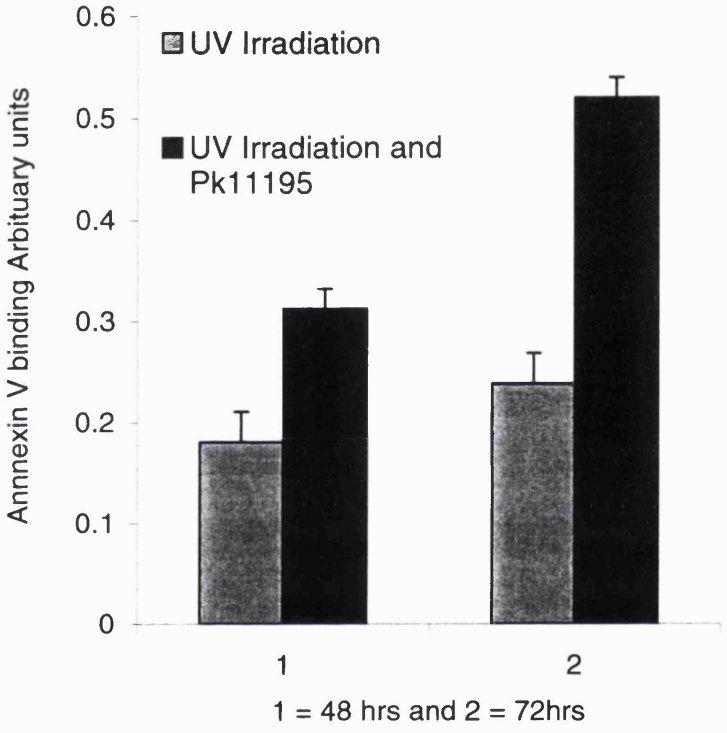


Figure 5f

Pk11195 increases the proprtion of human Tfk-1 cells that bind annexin V at 48 and 72 hrs following a 5 minute exposure to UV irradiation.

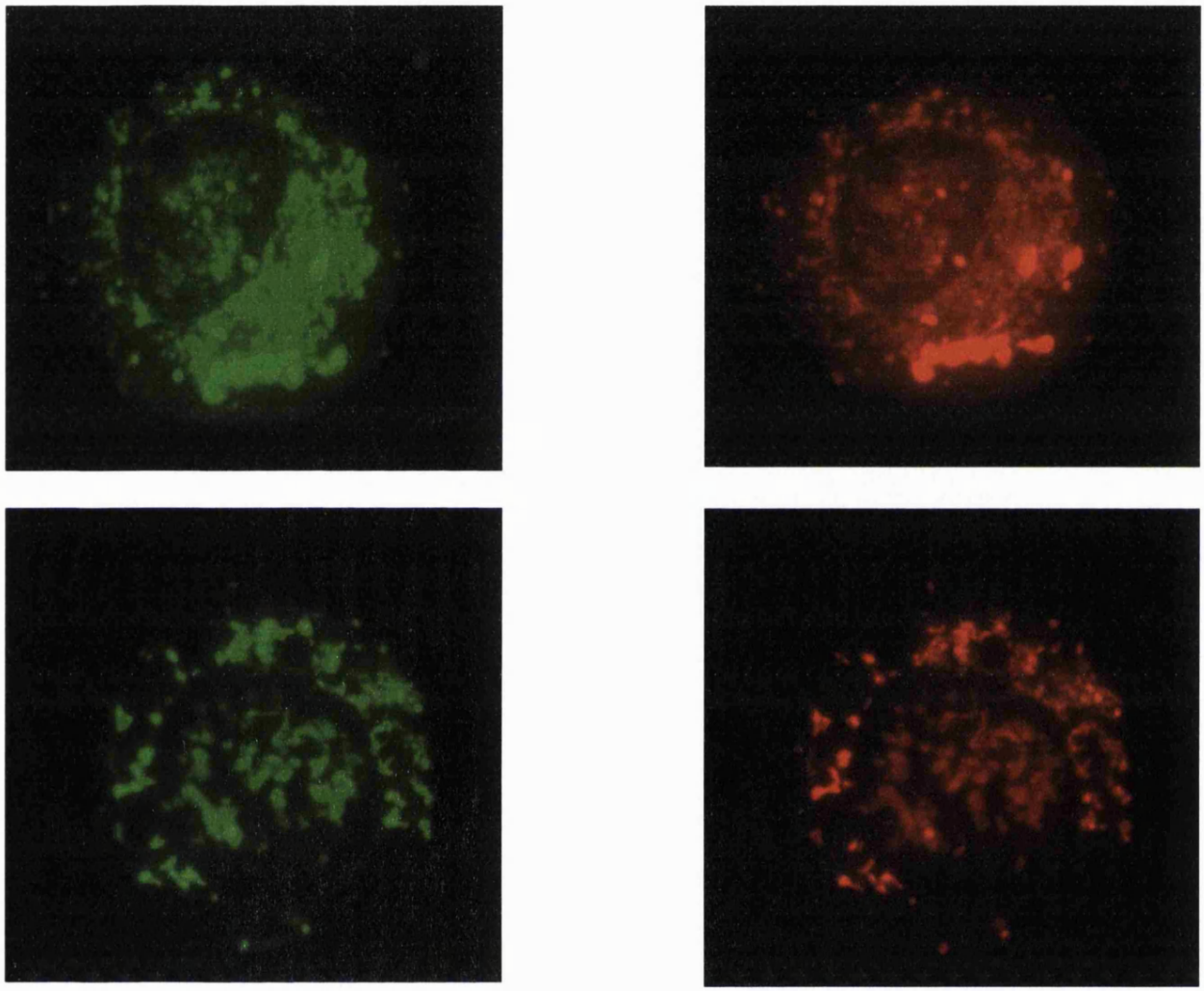


Figure 5g

Cholangiocarcinoma cells Egi-1 (top) and Tfk-1 (bottom) express the mBzR as shown by the indentical uptake of the mBzR probe NBD FGIN-1-27 and mitochondrial probe CMXRos.

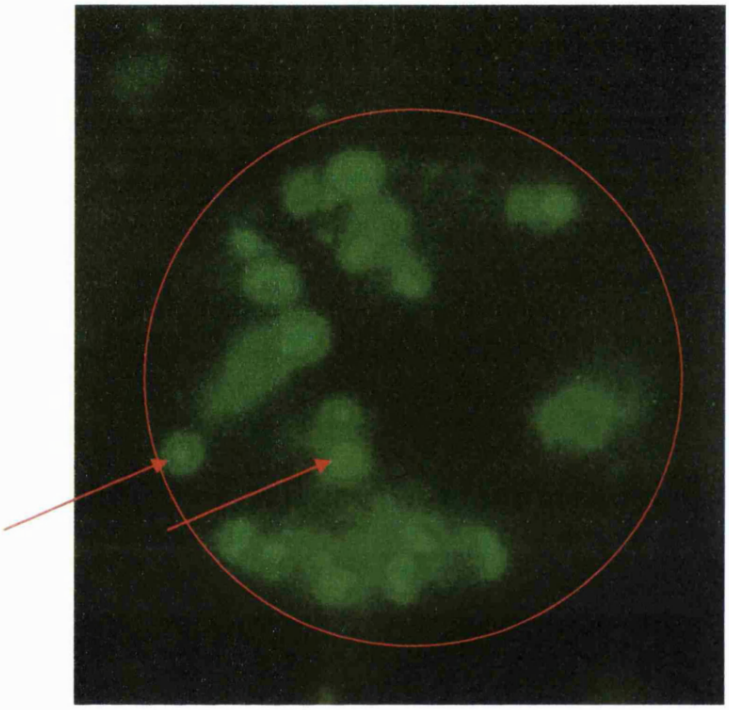


Figure 5h

Image showing the mitochondrial uptake (arrows) of the probe in a tumour cell obtained from the xenografts of an animal injected with NBD FGIN-1-27 analogue.

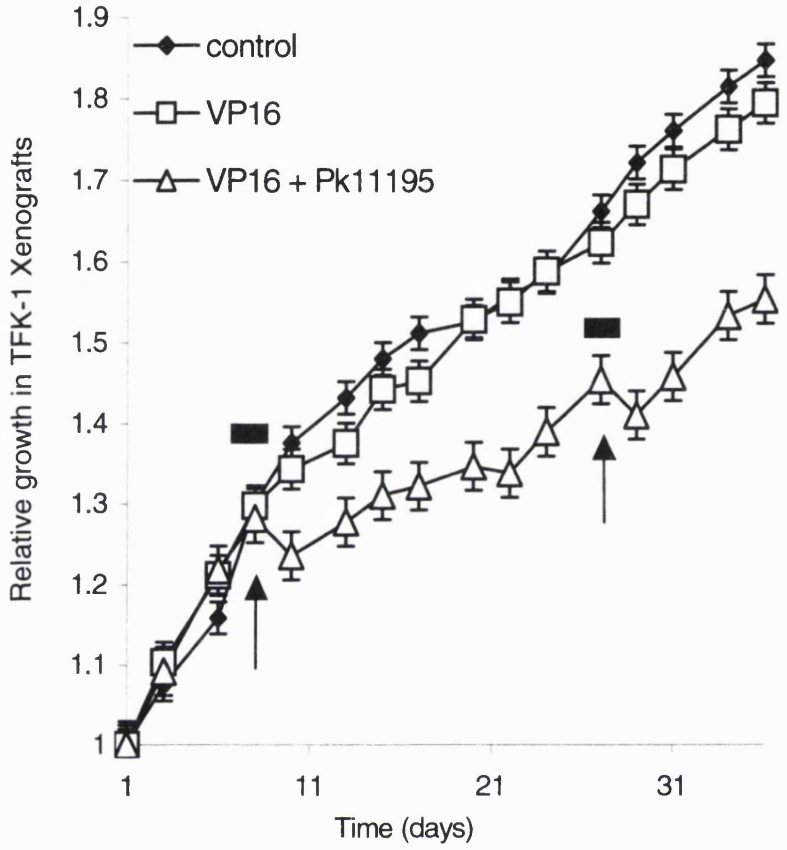


Figure 5i

Growth of Tfk-1 xenografts on SCID-NOD mice in treatment and control group over 35 days.

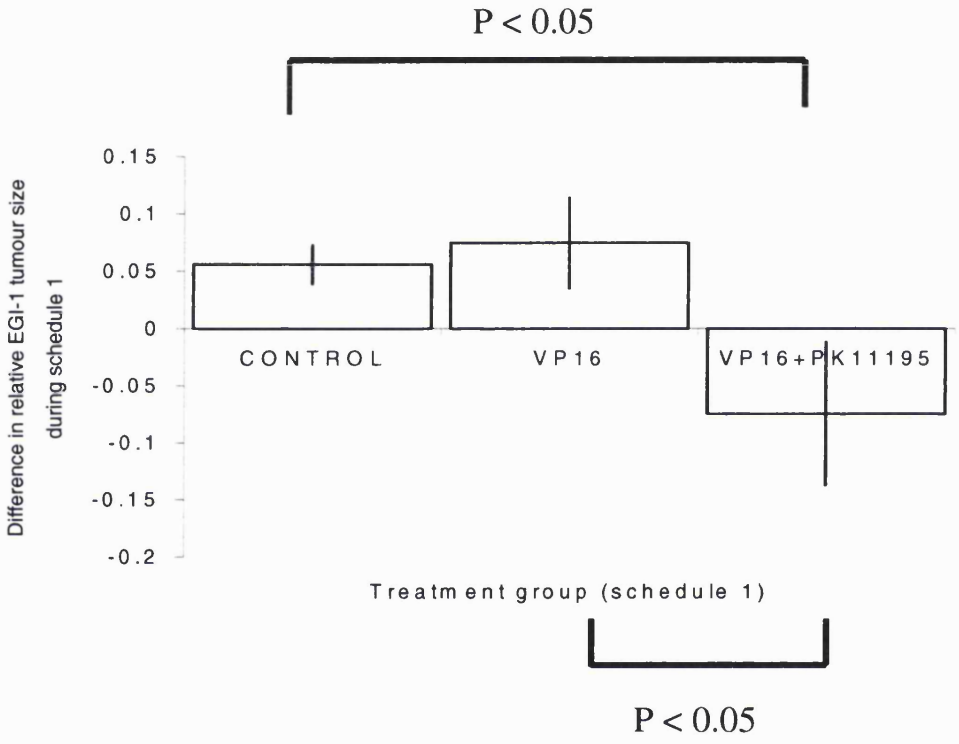


Figure 5j

Difference in Egi-1 xenograft growth over the 72 hour period following the 1st treatment (shedule 1)

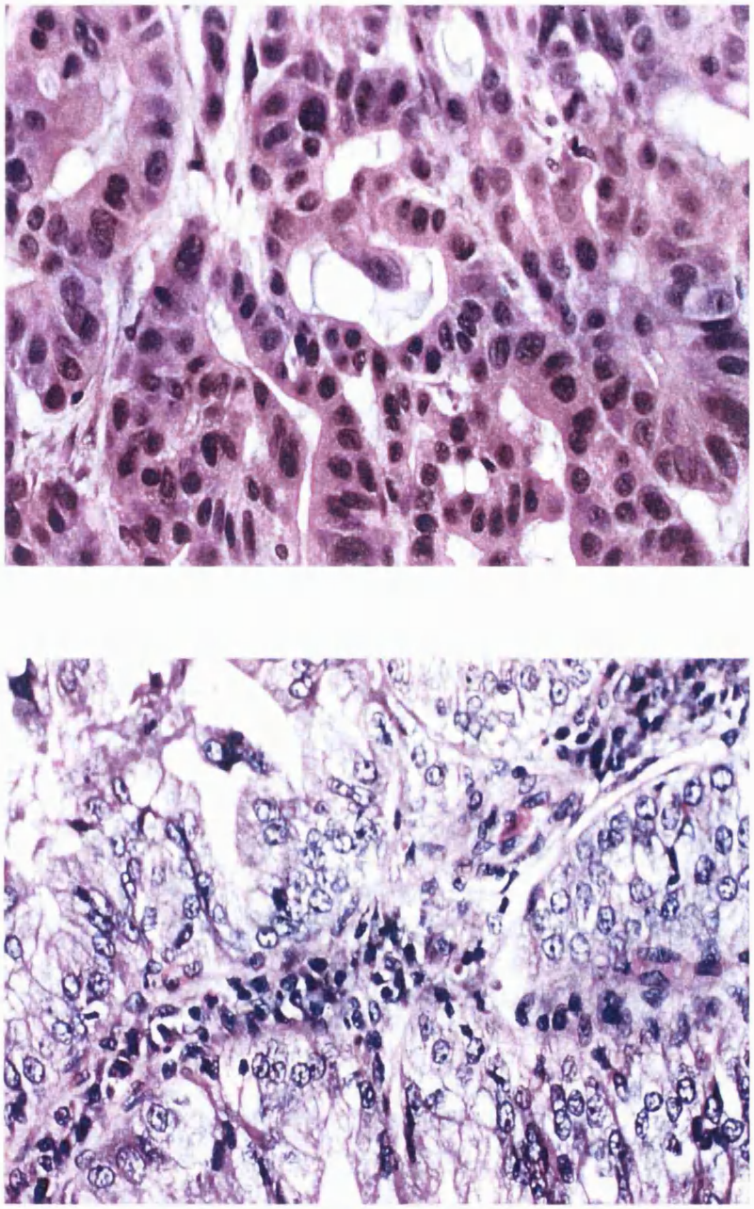


Figure 5 k

H & E staining of cholangiocarcinoma cell line Tfk-1 (upper) and Egi-1 (lower) xenograft on SCID/NOD mice(x 80).

5.5 Discussion

The factors leading to the poor response of cholangiocarcinoma to therapies other than curative surgery is poorly understood. This is mostly due to a lack of studies into the biology of this cancer.

The findings from the previous chapter demonstrate that human cholangiocarcinoma cells when treated with DNA damaging agents *in vitro* are resistant to apoptosis induction. The reasons why cholangiocarinoma cells fail to undergo apoptosis after DNA damamge is unknown. Antiapoptotic proteins such as Bcl-2, Mcl-1 and Bcl-X_L are well known because of their ability to block the induction of apoptosis in cancer cells following DNA damage (Boise et al 1993;Chao et al.1995b;Ibrado et al.1996b;Ibrado, Huang, Fang, & Bhalla 1996a;Reed 1997d;Yang & Korsmeyer 1996). Cholangiocarcinoma cells have the antiapoptotic proteins Mcl-1 and Bcl-X_L within their cytoplasm and on their mitochondria, whether these important central apoptosis regulating proteins are responsible for the poor response of cholangiocarcinoma cells to DNA damage has not been previously shown.

To study the part played by the mitochondria and its associated apoptosis inhibiting proteins, the mBzR and bcl-2 anatagonist Pk11195 (Hirsch et al. 1989) was used in the *in vitro* and *in vivo* experiments performed in this chapter. Apoptosis was measured at single cell level as described in chapter 2. The magnitude and rate of apoptosis following the treatment of human cholangiocarcinoma cells *in vitro* with chemotherapy, UV or X–ray irradiation was increased by the simultaneous addition of the Bcl-2 and mBzR antagonist Pk11195. The *in vivo* experiments in an identical way demonstrate that human cholangiocarcinoma xenograft growth was inhibited by the

simultaneous treatment of mice with Vp16 and Pk11195. In as much as it was possible to show using the immunofluorescent probe NBD FGIN-1-27 that these molecules reach the xenografts after intraperitoneal administration, it was not possible to demonstrate whether the apparent *in vivo* efficacy of Pk11195 was entirely apoptosis or indeed another mechanism. The ability of Pk11195 to significantly alter in this time and dose dependent manner the *in vitro* response of the human cholangiocarcinoma cell lines to DNA damage is consistent with a direct apoptosis enhancment. Judging from the co-localisation of the analogue and mitochondrial probe CMXRos this property may largely be dependent on its ability to localise to the mitochondria. The exact mechanism through which Pk11195 is capable of altering the mitochondrial response is not yet fully understood, but may involve changing the threshold required for mitochondrial pore opening (Hirsch, Marzo, & Kroemer 1997).

The ability of 75μM Pk11195 to sensitize Bcl-X_L and Mcl-1 expressing human cholangiocarcinoma cells to apoptosis confirms that these antiapoptotic proteins are important intracellular regulators of apoptosis in cholangiocarcinoma cells. Through their affects on mitochondrial response to the induction of apoptosis these proteins are likely to be contributing to poor responses to chemotherapy and radiotherapy. Futher detailed animal studies are required to outline fully the mechanism of action of Pk11195 and its side effects. The future may see the use of this or similar molecules in the treatment of solid cancers like cholangiocarcinoma.

CHAPTER 6 Overview and Discussion

The work contained in this thesis has attempted to take forward our understanding of cholangiocarcinoma, a relatively rare form of cancer with a poor prognosis. Surgery, chemotherapy, radiotherapy or a combination of each are the treatment options for patients with cholangiocarcinoma. Surgery is curative in a minority of patients where the diagnosis is made early with careful evaluation and selection of cases both preoperatively and intraoperatively. In the past, the overall results of treatment of bile duct cancer were unsatisfactory, with the majority of patients dead within one year from liver failure, tumour recurrence and sepsis. More aggressive surgical procedures that obtain tumour free margins have resulted in a more favourable survival in select group of patients with stage I and II disease. 5 year survivals of 9-18% and 20-30% in proximal and distal cholangiocarcinomas respectively have been reported (Baer et al. 1993;Bengmark, Ekberg, Evander, Klofver-Stahl, & Tranberg 1988;Broe & Cameron 1981b;Cameron, Pitt, Zinner, Kaufman, & Coleman 1990b;Casavilla et al. 1997b;Gores 2000;Helling 1994;Nimura et al. 1998;Schoenthaler et al.1994).

Chemotherapy and radiotherapy induce apoptosis in responsive cells (Eastman 1993;Hannun 1997). Apoptosis resistance is a major factor contributing to the failure of cytotoxic therapy in the treatment of solid tumours (Dive 1997b;Volm 1998a). Retrospective and prospective studies have failed to demonstrate any survival benefit for chemotherapy and/or radiotherapy with CCA (Bukowski, Leichman, & Rivkin 1983b;Hejna, Pruckmayer, & Raderer 1998;Leung, Guiney, & Das 1996;Leung & Kuan 1997b;Pazdur et al.

1999b;Poplin et al.1999a;Sanz-Altamira et al.1998a). The factor(s) responsible for this poor response to treatment are unknown.

In the first part of this thesis (chapter 3) the expression of some well characterized antiapoptotic proteins Bcl-2, Bcl-X_L and Mcl-1 was investigated in human cholangiocarcinoma tissue. Human cholangiocarcinoma tissue used in the study comprised archival paraffin embedded cholangiocarcinoma tissue from resected tumours and human cholangiocarcinoma cell lines. Using a combination of immunohistochemistry and immunofluoresence as the methods of protein detection, the antiapoptotic proteins Bcl-X_L and Mcl-1 were found to be co-expressed not only by the cholangiocarcinoma cells, but also by the normal biliary cells studied. Bcl-2 protein was not detected in either normal biliary epithelium nor cholangiocarcinoma cells. Using immunofluoresence it was demonstrated these antiapoptotic proteins were co-localised to intracellular organelles such as the mitochondria (Okaro et al. 2001). The expression of the antiapoptotic protein Bcl-2 in preserved human cholangiocarcinoma tissue has been studied by other groups but with conflicting results (Arora et al.1999b;Charlotte et al. 1994a;Okaro et al. 2001). Despite using identical techniques Bcl-2 protein expression was not found in cholangiocarcinoma cells. These results will need confirmation by other techniques.

A novel finding was the co-expression of the antiapoptotic proteins Bcl-X_L and Mcl-1 by cholangiocarcinoma cells. The expression of these proteins by human cholangiocarcinoma cells has no been previously reported and their biological importance requires to be investigated. However, Bcl-2 and Bcl-X_L proteins may have a reciprocal regulation of apoptosis (Boise et al.1993), which

may way explain why some cholangiocarcinoma cells express the Bcl-2 protein, while others express the Bcl-X_L protein. Antiapoptotic protein expression would therefore appear to be a recognised feature of this disease, although the molecular mechanisms or stimulus responsible for the expression of these antiapoptotic proteins by cholangiocarcinoma cells remains to be defined. *In vitro* and *in vivo* studies in a variety of haematological and solid tumours but not cholangiocarcinoma have shown a direct correlation between the expression of antiapoptotic proteins and resistance to chemotherapy induced apoptosis. Whether antiapoptotic proteins expressed by cholangiocarcinoma cells are performing an identical role and contributing to the poor levels of response to chemotherapy and radiotherapy in patients with cholangiocarcinoma, has yet to be established.

An interesting finding of the immunohistochemistry performed on the resected specimens that contained 'normal' liver tissue was that the cholangiocytes (epithelial cells) lining the unaffected bile duct radicals within the liver substance also stained positive for both Bcl-X_L and Mcl-1 proteins but not Bcl-2. This was a consistent finding in all the specimens. In order to verify these findings, fresh cholangiocytes were collected at ERCP (for benign disease) from 10 different patients, prepared and stained. An identical pattern of protein expression by these cells was once again detected. There was no obvious difference in the pattern or intensity of staining observed between the benign and malignant cells. One possible explanation why cholangiocytes express Bcl-X_L and Mcl-1 is that such cell survival proteins are required to maintain tissue homeostasis within the biliary tree. Such a process may be a necessary factor that limits excessive cell loss that can result from exposure of cholangiocytes to toxic

bile (bile acids/salts, xenobiotics, drugs, heavy metals etc). These substances are known to be toxic to epithelial cells in other parts of the alimentary tract such as the hepatocytes, lower oesophagus and the colon inducing apoptosis and even cancer formation (Benz et al. 2000;Bernstein et al. 1999a;Bernstein et al. 1999b;Ransford & Jankowski 2000;Schlottman et al. 2000;Webster, Usechak, & Anwer 2002). The ability of bile constituents to induce Bcl-X_L and Mcl-1 expression in cholangiocytes would be of interest. The prevention of bile induced cholangiocyte apoptosis afforded by the expression of these antiapoptotic proteins may avoid epithelial cell loss, which would lead to ulceration, scarring and fibrosis of the single layered bile ducts. Down-regulation of antiapoptotic proteins leading to increased apoptosis, cell loss and bile duct loss has been suggested to occur in primary biliary cirrhosis (PBC) (Graham et al. 1998;Iwata et al. 2000). The bile acid ursodeoxycholic acid used in the treatment of PBC has been found to delay progression to severe fibrosis and cirrhosis, the mechanism of action could be via the modulation of apoptosis (Paumgartner & Beuers 2002;Rodrigues & Steer 2001).

Immunohistochemistry and immunofluorescence was used in chapter I to detect the presence of antiapoptotic proteins and to determine their site of expression. Data on the quantity of proteins expression could be obtained from western blotting or the mRNA studies of northern blotting or PCR.

The second stage of the thesis (chapter 4) set out to test the hypothesis that antiapoptotic proteins such as Bcl-X_L and Mcl-1 expressed by human cholangiocarcinoma cells may be altering the apoptosis threshold in cholangiocarcinoma cells in response to chemotherapy and radiotherapy.

Two human cholangiocarcinoma cell lines (Egi-1 and Tfk-1) were treated with chemotherapy, UV and radiotherapy *in vitro* and dose response curves were generated. The Experiment involved treating human cholangiocarcinoma cells with various doses of either chemotherapeutic agents or of radiotherapy with sampling performed every 24 hours up to maximum of 96 hours. The dose response curves demonstrate that the human CCA cells are resistant to the apoptosis inducing effects of chemotherapy and radiotherapy, but donot establish whether the antiapoptotic proteins are responsible for the resistance to therapy.

A number of such mechanisms for chemotherapy resistance are well described (Volm 1998a). They range from extrusion pumps that prevent the chemotherapeutic drugs reaching their target (nucleus) by blocking drug accumulation within the cell such as the multi-drug protein P-glycoprotein (p-gp) or detoxifying mechanisms like the glutathione redox system (Chu 1994). Others include mitochondrial dysfunction usually resulting from certain proteins interacting and altering ionic channel function, Bcl-2 and Bcl-X_L are well known examples. The experiments performed in the second part of chapter 4 focused on investigating whether cholangiocarcinoma cells possess mechanisms that will allow them evade apoptosis. The human CCA cell lines Tfk-1 and Egi-1 were in the first instance investigated and found not to express the cell surface protein p-gp. This expression of this protein has not been previously studied in cholangiocarcinoma cells. With cells not expressing p-gp, drug accumulation within the cell is likely to occur permitting drug target interaction. The cell cycle changes that occurred upon exposure to the drugs indirectly confirm that drug target interaction took place.

Cell cycle studies following chemotherapy even at low doses showed a sustained G2/M arrest. This was both time and dose dependent. This confirms that chemotherapy does produce cell cycle arrest of the cholangiocarcinoma cell lines but that there is a delay or failure of cells to either re-enter the cycle or undergo apoptosis which may be important in allowing for DNA repair (Funk 1999;Powell & Abraham 1993;Teyssier et al. 1999).

The manner in which the cells responsed to the mitochondrial oxidising agent diamide rapidly undergoing mitochondrial depolarisation was in sharp contrast to the response observed following chemotherapy and/or radiotherapy. Diamide acts directly on the mitochondria, it's ability to induce rapid apoptosis in Tfk-1 and Egi-1 in contrast to chemotherapy and radiotherapy demonstrates that there is block in the apoptosis signaling pathway in the human cholangiocarcinoma cells studied located between nuclear generated signals and their interaction with the mitochondria.

The Bcl-2 family of proteins are well known for their ability to attenuate cellular responses to apoptosis induction both *in vitro* and *in vivo.* These proteins directly and indirectly interact and altering the behaviour of the multi-protein megachannel complex of the mitochondria otherwise known as the PTPC. It is unknown whether of Bcl-X_L and Mcl-1 proteins co-expressed by cholangiocarcinoma cells by interacting with the megachannel of the mitochondria in these cells may be attenuating response to treatment and leading to resistance.

There are several strategies available which could help to establish the role of Bcl-2 or Bcl-X_L proteins in tumour cells. Gene silencing techniques such as antisense oligonucleotide therapy targeted against the Bcl-2 gene, have been used

to reduce protein levels both *in vitro* and *in vivo*, restoring chemosensitivity and facilitating apoptosis in lymphoma cells (Ackermann et al. 1999;Chen, Wang, & Huang 1996;Kitada et al.1994;Webb et al. 1997). Similar results have been obtained using antisense against the Bcl-X_L protein in keratinocytes (Taylor et al. 1999). Antisense therapy proved a break through in the field of cell biology enabling scientists to downregulate protein levels in order to understand function, however, the size and resulting impermeable characteristics of these molecules together with the cost limits their wider use.

Recently, there has been a growing interest in the use of a new class of response modifying drugs (small molecules) that specifically target the mitochondria, and produce functional antagonism of the antiapoptotic Bcl-2 members. The mitochondrial benzodiazepine receptor antagonist Pk11195 reverses the effects of Bcl-2 on apoptosis induction following chemotherapy in a leukaemia cell line (Hirsch et al. 1998c). Another such molecule, arsenic trioxide, has shown encouraging results in the treatment of patients with acute promyelocytic leukemia (Soignet, Maslak, Wang, Jhanwar, Calleja, Dardashti, Corso, DeBlasio, Gabrilove, Scheinberg, Pandolfi, & Warrell, Jr. 1998) where it directly induces the mitochondrial release of cytochrome c through its interaction with the PTPC (Larochette et al. 1999). 5 δ aminolaevulinic acid (5- ALA) is used as a pro-drug to generate the endogenous ligand of the mBzR, protoporphyrin IX, in photodynamic therapy (PDT) (Svanberg et al. 1996;van den Boogert et al.1998;Verma et al.1998). Although, the exact mechanism by which such agents overcome the protection afforded by antiapoptotic protein expression is yet to be

shown, there is evidence to suggest that they interfere with mitochondrial ATP synthesis (Vander Heiden et al. 1999c).

In chapter 5 the Bcl-2 antagonist and mitochondrial benzodiazepine receptor antagonist Pk11195 was used in experiments with cholangiocarcinoma as a response-modifying agent. At non-cytotoxic doses (no effect on the baseline apoptosis) this drug had a marked chemo and radiosensitising effect on cholangiocarcinoma cell both *in vitro* and *in vivo*. This mitochondrial-targeting agent was capable of lowering the apoptosis threshold resulting in facilitated and accelerated response of cholangiocarcinoma cells to chemotherapy and radiotherapy under experimental conditions. The results demonstrate that antagonising the effects of Bcl-X_L and Mcl-1 proteins within the cell can significantly increase the apoptosis in cholangiocarcinoma cells. The evidence for the first time supports a link between the expression anti-apoptotic proteins and apoptosis resistance in cholangiocarcinoma. The reference of these findings to the treatment of patients with cholangiocarcinoma remains to be established but mitochondrial targeting small molecule apoptosis sensitisers such as Pk11195 in combination with conventional cytotoxic therapy is a new and exciting possibility in the treatment of solid cancers.

FUTURE RESEARCH

The thesis has studied the possible role for the mitochondrial targeting therapy in treatment of cholangiocarinoma. Future studies should aim to clarify the role of the mitochondria in the control of apoptosis in cholangiocarcinoma cells

Through research our understanding of apoptosis is rapidly changing and links are being established between scientific experimental studies and clinical trials. New therapies tailored to individual patients and their tumours will emerge with scientists and clinicians working more closely together.

IMMUNOSTAINING USING DAKO DUET

1	Dewax in xylene	lOmins
2	Take through alcohol	
3	Block endogenous peroxidase with hydrogen peroxide in methanol	10 mins
	10 mins hydrogen peroxide in 500 mIs methanol	
4	Wash in running water	
5	Appropriate antigen retrieval - no pretreatment,, pressure cooker, microwave oven or trvpsinkhvmotrypsin (0.05 gins + 0.05 gins in 100 mIs 0.1% calcium chloride p1-17.8).	
6	Wash in running water	
7	Ring sections with PAP pen when laying out in staining trays ENSURE the sections do not dry out. Wash with Tris buffered saline (TBS).	
8	10% normal goat serum	10 mins
9	Apply primary antibody at optimal dilution.	60 mins
10	Wash in several changes of lBS	
11	Apply seconda~ antibody at 1/100 dilution (Bottle C in Duet kit)	30 mins
12	Prepare avidin biotin complex at a dilution of 1 + 1/100 around 30 minutes before required. (bottles A and B).	
13	Wash slides in several changes of lBS	
14	Apply avidin biotin complex	30niins
15	Wash in several changes of TBS	
16	Apply DAB solution	lOmins
17	Wash off with lBS. put slides in staining racks	
18	Wash in running water	
19	Counterstain with Haematoxylin, diff and blue.	
20	Dehydrate clear and mount.	

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