APOPTOSIS AND EXPRESSION OF APOPTOSIS-REGULATING PROTEINS IN HEPATOCELLULAR, GALLBLADDER AND PANCREATIC CARCINOMAS

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Academic Dissertation to be presented with the assent of the Faculty Medicine, University of Oulu, for public discussion in the Auditorium of the Department of Pharmacology and Toxicology, on April 28th, 2000, at 12 noon.

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Apoptosis is a biochemically regulated mechanism leading to the destruction of an individual cell. An inadequate apoptosis is partly responsible for uncontrolled survival of malignantly transformed cells and formation of cancer. The growth of a tumor depends on the proliferative capacity and destruction of tumor cells either through apoptosis or necrosis. In this work, the extent of apoptosis and the expression of apoptosis-regulating proteins were studied by 3-end labeling of fragmented DNA (TUNEL) and immunohistochemistry in a set of 166 tissue samples consisting of 33 HCCs, 39 gallbladder carcinomas, 7 gallbladder dysplasias and 87 pancreatic carcinomas. In addition, p53 protein and P-glycoprotein expression was studied immunohistochemically.

The extent of apoptosis was estimated by using apoptotic index, defined as a percentage of apoptotic cells in the entire tumor cell population. The present results show an average apoptotic index of 0.73% in HCC, 0.68% in gallbladder and 0.69% in pancreatic carcinoma. Bcl-2 positivity was found in only 3% of the HCCs, 10% of gallbladder and 13% of pancreatic carcinomas. Bax positivity was seen in all of the gallbladder and pancreatic carcinoma cases. Mcl-1 positivity was found in 87% of gallbladder and 86% of pancreatic tumors. The apoptotic index in bcl-2 positive cases was lower (0.35%) than in cases showing no immunoreactivity (0.64%) in pancreatic tumors (P = 0.013). Apoptotic index was higher in pancreatic tumors with strong bax immunoreactivity (0.70%) than in other cases (0.34%) (P = 0.002). Caspase 3, 6 and 8 expression was found in 92%, 92% and 73% of HCC, 95%, 77% and 77% of gallbladder carcinoma and 80%, 80% and 74% of pancreatic carcinoma cases, respectively. p53 positivity was found in 23% of hepatocellular, 57% of gallbladder and 41% of pancreatic carcinomas. P-glycoprotein was observed in 65% of the HCCs. Patients with Pgp positive tumors had a significantly shorter disease-free interval than those with Pgp negative tumors (P<0.05).

To evaluate the growth potential of HCC and pancreatic carcinoma, a growth index from the scores obtained for apoptosis, necrosis and cell proliferation was designed. Patients with a high degree of proliferation relative to the degree of necrosis and apoptosis (i.e. had a positive growth index) in HCC lesions had a significantly shorter survival (P = 0.004) and disease-free interval after operation (P = 0.019) than those with a tumor predominated by apoptosis and necrosis. Results were in line with HCC in pancreatic carcinoma, but the association did not reach statistical significance (P=0.09).

According to the results the extent of apoptosis was similar in HCCs, gallbladder and pancreatic carcinomas. These tumors also showed here a similar expression pattern of the bcl-2 family of proteins and caspases. None of the individual parameters associated significantly with apoptosis except for bcl-2 and bax in pancreatic carcinoma, neither was there any association between p53 and P-glycoprotein expression and apoptosis. Calculation of a growth index might be helpful in assessing the prognosis of patients with tumors with a scant stroma, such as HCC.

Keywords: p53, bcl-2, caspases, P-glycoprotein.
To Sami and Julia
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Oulu, March 2000

Nina Turunen
Abbreviations

ADP  Adenosine diphosphate
AIDS  Acquired immunodeficiency syndrome
AIF   Apoptosis inducing factor
ALS   Amyotrophic lateral sclerosis
Apaf-1 Apoptotic protease activating factor-1
ATP   Adenosine triphosphate
bax   Bcl-2 homologous antagonist x
bcl-2  B-cell leukemia/lymphoma-2
BH    Bcl-2 homology
CARD  Caspase recruitment domain
CB    Citrate buffer
ced   Cell death abnormal
cFLIP cellular FLICE
Crm A Cytokine response modifier A
CTL   Cytotoxic lymphocyte
DAB   3,3-diaminobenzidine tetrahydrochloride
Daxx  Death domain associated protein
DD    Death domain
DED   Death effector domain
DISC  Death inducing signalling complex
DNA   Deoxyribonucleic acid
FADD  Fas-associated death domain
FasL  Fas ligand
FLICE Fadd like ICE
FLIP  FLICE-inhibitory protein
HBV   Hepatitis B virus
HCC   Hepatocellular carcinoma
HCV   Hepatitis C virus
hid   Head involution defective
HPF   High power field
IAP   Inhibitor of apoptosis protein
ICE   Interleukin-1β-converting enzyme
JNK  c-Jun-N-terminal kinase
kDa  kilodalton
K-ras  Kirsten-ras
mcl-1  Myeloid cell leukemia 1
mdm2  Mouse double minute 2
MDR  Multidrug resistance
MORT  Mediator of receptor-induced toxicity
mRNA  messenger ribonucleic acid
MRP  Multidrug resistant protein
NF-κB  Nuclear factor-kappa B
NuMA  Nuclear matrix protein
PARP  Poly(ADP-ribose) polymerase
PBS  Phosphate-buffered saline
PCNA  Proliferating cell nuclear antigen
Pgp  P-glycoprotein
PIG  p53 inducing gene
RAIDD  RIP associated ICH-1/CED-3 homologous protein with a death domain
Rb  Retinoblastoma
RIP  Receptor interacting protein
rpr  Reaper
SLE  Systemic lupus erythematosus
TNF  Tumor necrosis factor
TNF-R1  Tumor necrosis factor receptor 1
TRADD  TNF-R1-associated death domain protein
TRAIL  TNF-related apoptosis inducing ligand
TRAMP  TNF-related apoptosis mediating protein
TUNEL  Terminal deoxytransferase-mediated dUTP nick-end labeling
UV  Ultraviolet
vFLIP  viral FLICE
List of original papers

This thesis is based mainly on the following articles, which are referred to in text by their Roman numerals. In addition, some unpublished data is presented.


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1. Introduction

Apoptosis is a genetically controlled process in which individual cells are triggered to undergo self destruction without injuring neighboring cells or causing any inflammatory reaction (Kerr et al. 1994, White 1996, Cummings et al. 1997). Apoptosis is designed to eliminate excess and aged cells that have lost their functional importance. The activation of this controlled, internally programmed chain of events is involved in morphogenesis of embryonic tissues as well as in homeostasis of adult organs and tissues (Huppertz et al. 1999). Inappropriate apoptosis is involved in the pathogenesis of diseases such as AIDS, neurodegenerative disorders, stroke, myocardial infarction and cancer (Salvesen 1999, Saraste et al. 1997). The growth of a tumor depends on the proliferative capacity and destruction of the tumor cells either through apoptosis or necrosis. An inadequate apoptosis is partly responsible for uncontrolled accumulation of malignant cells and formation of cancer (Reed 1994).

Several oncogene and tumor suppressor gene products are involved in the regulation and execution of apoptosis. Some of these activate apoptotic cell death while others inhibit it. For instance, p53 halts the cell cycle and allows DNA repair to take place. If the repair fails, p53 protein triggers apoptosis in the damaged cells. Because dysfunctional p53 is unable to perform these functions, there is an increased mutational frequency in the affected cell population and a malignant tumor may develop (Lane 1992a, Greenblatt et al. 1994). The bcl-2 family is a group of apoptosis regulating genes comprising both anti- and proapoptotic members such as bcl-2, mcl-1 and bax. The amount of apoptosis inhibiting and promoting dimers determines a cell’s susceptibility to die. Bcl-2 family members act upstream of caspases, a family of cysteine proteases, which activate themselves in a cascade-like fashion, leading to fragmentation of proteins resulting in disassembly of the cell (Tsujimoto 1998, Wolf & Green 1999).

The liver, biliary apparatus and pancreas constitute a both anatomically and physiologically connected unit which originates from the same region, the foregut, during embryonic development (Sadler 1990). Hepatocellular, gallbladder and pancreatic carcinomas are rarely discovered early in their course and survival rates for these tumors are extremely poor. The incidences for these malignancies have been relatively stable during the last years (Engeland et al. 1995).

The present study was designed to investigate the extent of apoptosis and its association to apoptosis-regulating proteins and survival of the patients in these high-
mortality malignancies. In addition, tumor proliferation as determined by the PCNA index and mitotic count, and the extent of necrosis as determined by morphology from hematoxylin-eosin stained slides, were studied in the tumors. p53 expression in the tumors was also studied. Additionally P-glycoprotein was determined in HCCs. To evaluate the growth potential of HCCs and pancreatic carcinomas, a growth index from the scores obtained for apoptosis, necrosis and cell proliferation was designed.
2. Review of the literature

2.1. Epidemiology

2.1.1. Hepatocellular carcinoma (HCC)

Primary liver cancer constitutes the most common visceral malignant tumor and in some populations it is the most common cancer overall. Annual incidence rates vary from 3 to 120 cases per 100,000 population within different geographic areas (Akriviadis et al. 1998). HCC ranks eighth in frequency among cancers worldwide (Schafer & Sorrel 1999). HCC is irregularly distributed in the world, for the most part following the prevalence of the HBV (Schafer & Sorrel 1999). While highest incidence rates are found in Asia, Africa and southeast China, the countries of North and South America and northern and central Europe have lower ones (Wands & Blum 1991, Schafer & Sorrel 1999). Within each geographic area, blacks have approximately fourfold higher rates than whites (Crawford 1999). An increasing incidence of HCC has been noted worldwide (Craig et al. 1988). Men are afflicted at least twice as often as women (Schafer & Sorrel 1999). Most cases are seen in patients over the age of 50 years, but this tumor can occur in younger individuals and even in children (Barwick & Rosai 1996).

In 1995 liver cancer was diagnosed in 133 males and 86 females in Finland (Finnish Cancer Registry 1997). When leading primary sites of cancer in males were investigated, liver cancer was in 14th place (not listed in females) (Finnish Cancer Registry 1997). Between the years 1985-1994 2035 new liver cancer diagnoses were made in Finland. The average age of patients was 69 and the ratio between men and women was 1:1 (Dickman et al. 1999).

Patients with liver cancer have an extremely poor prognosis. The overall median survival of untreated HCC is about 4 months (Barwick & Rosai 1996). The 5-year relative survival rate in 1985-1994 in Finland was only 5% for males and 6% for females in all cases, and 16% (males) and 18% (females) with localized cancer. The survival rates for liver cancer have remained constant during the past years (Dickman et al. 1999).
2.1.2. Carcinoma of the gallbladder

Carcinoma of the gallbladder is more common in females, with a 3:1 ratio, and occurs most frequently in the sixth and seventh decade of life (reviewed by Levin 1999). Adenocarcinoma of the gallbladder is the fifth most common gastrointestinal malignancy (reviewed by Levin 1999). The incidence is high among American Indians and Hispanic Americans and among South and Central American Indians, relatively low among whites of European extraction, and very rare among blacks. In Europe, the rate is very high in Germany and surrounding central European countries, low in Mediterranean countries, and low and declining in Britain and Ireland (Albores-Saavedra & Henson 1986, Rosai 1996a).

74 new gallbladder cancer diagnoses were made in males and 166 in females in Finland in 1995 (Finnish Cancer Registry 1997). It came in 17th on a list of the leading primary sites of cancer in females (not listed in males) (Finnish Cancer Registry 1997). The total number of new gallbladder cancers in Finland between the years 1985-1994 was 2539 and the average age of the patients was 72. The number of cases among females was almost three times higher than among males (Dickman et al. 1999).

The prognosis of patients is poor and only a slight improvement in the survival rates is observable. Gallbladder cancer is only rarely discovered at a resectable stage, and the global mean 5-year survival is less than 5%, despite surgical intervention (Kyriacou 1999). In Finland the 5-year survival rates among patients with localized cancer was 28% in males and 38% in females (Dickman et al. 1999). In all cases including both males and females it was 8% (Dickman et al. 1999).

2.1.3. Carcinoma of the pancreas

In industrialized countries the annual age-adjusted incidence rates of pancreatic carcinoma range from 8.0 to 12.0 per 100 000 males and from 4.5 to 7.0 per 100 000 females (Solcia et al. 1997). The male preponderance is usually about 1.6:1 (MacMahon 1982), but on the other hand, in the United States there were slightly more cases in women than in men (Abrams 1999). In most third world countries the incidence rates vary from 1.0 to 2.0 per 100 000 people (Flanders & Foulkes 1996). Incidence and mortality rates are almost identical, since survival rates for pancreatic carcinoma are extremely low (Solcia et al. 1997). Pancreatic cancer is the fifth most common cause of cancer death in both men and women worldwide (Flanders & Foulkes 1996). Pancreatic carcinoma is characteristically a tumor of elderly individuals (MacMahon 1982).

Statistics from the year 1995 showed 306 new cases of pancreatic cancers in males and 350 in females in Finland (Finnish Cancer Registry 1997). Pancreas was the 9th most common primary site of cancer in males and 10th in females (Finnish Cancer Registry 1997). There were 6405 new pancreatic cancers in Finland between the years 1985-1994 and the average age of the patients was 70 years (Dickman et al. 1999). There were almost as many cases among males and females (Dickman et al. 1999). The incidences of pancreatic cancer have been relatively stable in the Nordic countries since the mid-1970s.
(Engeland et al. 1993). The average annual numbers of cases is predicted to increase from 1665 to 2138 in females between 2008-2012 (Engeland et al. 1995).

The prognosis of patients with pancreatic cancer is one of the worst among all cancers. The mean survival time of the untreated patient is 3 months, while the mean survival time after radical resection varies from 10 to 20 months (Solcia et al. 1997). The diagnosis in most cases is made when the tumor is relatively large (about 5 cm) and has extended beyond the pancreas (85% of the cases). The 5-year survival in 1985 to 1994 was only 2% in males and 3% in females. In localized cancer it was slightly better, 10% in males and 13% in females (Dickman et al. 1999).

### 2.2. Etiology

#### 2.2.1. Hepatocellular carcinoma (HCC)

Chronic liver disease is the leading associated factor with HCC. On a global basis, distribution of HCC is strongly linked with the prevalence of HBV and HCV infection (Schafer & Sorrell 1999). In high incidence regions the cancer often occurs between 20 to 40 years of age. In the Western world, where HBV is not prevalent, cirrhosis is present in 70 to 90% of HCC cases and it rarely occurs before age of 60 years (Akriviadis et al. 1999). The most common associations are chronic infection with HCV and alcohol (Schafer & Sorrell 1999). Mutations of the p53 gene are found in 25 to 50% of HCCs (Greenblatt et al. 1994). There is a wide geographical variation in the incidence of p53 mutations in HCC that depends on the presence or absence of two major risk factors; hepatitis B and C infections and dietary aflatoxin (Greenblatt et al. 1994, Wands & Blum 1991). Aflatoxin, produced by a contaminating mould, Aspergillus flavus, is highly carcinogenic and can readily contaminate food such as ground nuts or grain, especially when stored in tropical conditions. Aflatoxin may also act as a co-carcinogen with HBV (Akriviadis et al. 1998). In addition, p53 mutation in populations exposed to a high dietary intake of aflatoxin B1 is an etiological cause for HCC in some geographical regions like Mexico (Soini et al. 1996). In Europe and North America, where both of these risk factors are low or nonexistent, the incidence of HCC is lower and p53 mutations in liver cancer occur in only 10 to 25% of HCCs (Greenblatt et al. 1994, Volkmann et al. 1994). A large proportion of HCCs have been found to associate with one or another of the factors subsequently listed in Table 1 (Greenblatt et al. 1994, Barwick & Rosai 1996, Crawford 1999, Schafer & Sorrell 1999).
Table 1. Predisposing and associated factors for HCC.

<table>
<thead>
<tr>
<th>Factor</th>
<th>Cause</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cirrhosis</td>
<td>-alcoholic (60-70%)</td>
</tr>
<tr>
<td></td>
<td>-viral (10%)</td>
</tr>
<tr>
<td></td>
<td>-cryptogenic (10-15%)</td>
</tr>
<tr>
<td></td>
<td>-biliary disease (5-10%)</td>
</tr>
<tr>
<td></td>
<td>-primary hemachromatosis (5%)</td>
</tr>
<tr>
<td></td>
<td>-Wilson disease</td>
</tr>
<tr>
<td></td>
<td>-α₁-antitrypsin deficiency</td>
</tr>
<tr>
<td>Liver cell dysplasia</td>
<td></td>
</tr>
<tr>
<td>Adenomatous hyperplasia</td>
<td></td>
</tr>
<tr>
<td>Hepatitis B and C</td>
<td></td>
</tr>
<tr>
<td>Thorium dioxide exposure</td>
<td></td>
</tr>
<tr>
<td>Androgenic anabolic steroids</td>
<td></td>
</tr>
<tr>
<td>Progestational agents</td>
<td></td>
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<tr>
<td>α₁-antitrypsin deficiency</td>
<td></td>
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<tr>
<td>Tyrosinemia</td>
<td></td>
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<tr>
<td>Ataxia-telangiectasia</td>
<td></td>
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<tr>
<td>p53 mutations</td>
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<tr>
<td>Aflatoxins</td>
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<tr>
<td>Schistosomiasis</td>
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</table>

2.2.2. Carcinoma of the gallbladder

As discussed above, gallbladder cancer is concentrated in certain racial and ethnic groups. Epidemiological studies have suggested that there is genetic predisposition for the gallbladder cancer. Also racial intermixture may have an effect (Albores-Saavedra & Henson 1986). Carcinoma of the gallbladder seems to associate with gallstones, which are present in 74 to 92% of cases (reviewed by Levin 1999). This possible etiologic relationship is strengthened by the observation that there is a higher incidence of carcinoma in females, who have a higher incidence of gallstones than males. Gallstones are more frequent in the gallbladder than in the bile ducts, and gallbladder carcinoma is more common than bile duct carcinoma. There is also the matter of higher incidence of gallbladder carcinoma in some ethnic groups who at the same time have a high incidence of gallstones, and the low incidence in the groups with fewer gallstones (Albores-Saavedra & Henson 1986). The risk to develop gallbladder carcinoma increases simultaneously with gallbladder size (reviewed by Levin 1999). Obesity is a confounding factor (Kyriacou 1999). Cancer is more likely to occur in a case of a single large gallstone than multiple smaller ones (reviewed by Levin 1999). It seems that gallbladders containing stones or infectious agents develop carcinoma as a result of irritative trauma and chronic inflammation (Kyriacou 1999). Carcinogenic derivatives of bile acids may
also have an effect on the development of carcinoma (Kyriacou 1999). In addition, a correlation exists between p53 abnormalities and the development of gallbladder carcinoma. p53 protein expression was related to higher grades of malignancies (Diamantis et al. 1995). The K-ras mutation may also be an important factor in the early state of carcinogenesis (Hanada et al. 1996). In Asia pyogenic and parasitic diseases of the biliary tract are common. Symptoms are insidious and indistinguishable from the ones associated with cholelithiasis. Approximately 1% of cholecystitis patients undergoing cholecystectomy have an unexpected gallbladder cancer (reviewed by Levin 1999). Gallbladder carcinoma usually presents late in its course, after the tumor has spread (Kyriacou 1999). Conditions associated with an increased risk of gallbladder cancer are listed in Table 2 (Kyriacou 1999, Levin 1999, Rosai 1996a).

<table>
<thead>
<tr>
<th>Factor</th>
<th>Cause</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gallstones</td>
<td></td>
</tr>
<tr>
<td>Genetic abnormalities</td>
<td>-p53</td>
</tr>
<tr>
<td></td>
<td>-K-ras</td>
</tr>
<tr>
<td>Choledochal cysts</td>
<td></td>
</tr>
<tr>
<td>Gallbladder polyps</td>
<td>-Peutz-Jeghers syndrome</td>
</tr>
<tr>
<td>Anomalous pancreaticobiliary</td>
<td></td>
</tr>
<tr>
<td>junction</td>
<td></td>
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<tr>
<td>Industrial exposure to</td>
<td></td>
</tr>
<tr>
<td>carcinogenes</td>
<td></td>
</tr>
<tr>
<td>Porcelain gallbladder</td>
<td></td>
</tr>
<tr>
<td>High body mass index</td>
<td></td>
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<tr>
<td>Cholecystoenteric fistula</td>
<td></td>
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<tr>
<td>Adenomyomatosis</td>
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<tr>
<td>Gardner’s syndrome</td>
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<tr>
<td>Polyposis coli</td>
<td></td>
</tr>
</tbody>
</table>

**2.2.3. Carcinoma of the pancreas**

Environmental factors, including smoking, chronic alcohol intake and consumption of a high-energy diet rich in fats have been implicated in the development of pancreatic carcinoma (Flanders & Foulkes 1999). Strong positive correlations between the consumption of oil, fat, butter, beef, pork, sugar and eggs as risk factors for pancreatic carcinoma have been found (Flanders & Foulkes 1996). Also consumption of smoked food has appeared to be a risk factor (Flanders & Foulkes 1996). Incidence rates are several times higher in smokers than nonsmokers (Flanders & Foulkes 1999). A previous history of pancreatitis, both acute and chronic, is a risk factor for pancreatic adenocarcinoma (Bansal & Sonnenberg 1995). An association between diabetes mellitus and pancreatic carcinoma has been noticed, although there has been some uncertainty as
to whether diabetes in the majority of pancreatic carcinoma patients is a symptom or a cause of the tumor (Everhart & Wright 1995). Other possible factors include cystic fibrosis, previous partial gastrectomy and blood group A. Suggestions have also been made that genetic factors could be important in a minority of cases. There are inherited symptoms, such as familial atypical mole-multiple melanoma, familial breast and ovarian cancer syndromes and Peutz-Jeghers syndrome associating with pancreatic adenocarcinoma (Flanders & Foulkes 1996). Mutations and/or accumulation of p53 are detected in about half of the pancreatic carcinoma cases, mutations of the K-ras oncogene in over 80% of the cases (Almoguerra et al. 1988, Sinicrope et al. 1994). Possible risk factors for pancreatic carcinoma are listed in Table 3 (Flanders & Foulkes 1996, Rosai 1996b, Solcia et al. 1995).

Table 3. Risk factors for pancreatic carcinoma.

<table>
<thead>
<tr>
<th>Factor</th>
<th>Cause</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dietary reasons</td>
<td></td>
</tr>
<tr>
<td>Chronic alcohol consumption</td>
<td></td>
</tr>
<tr>
<td>Smoking</td>
<td></td>
</tr>
<tr>
<td>Chemicals</td>
<td></td>
</tr>
<tr>
<td>Pancreatitis</td>
<td></td>
</tr>
<tr>
<td>Diabetes mellitus</td>
<td>-inherited syndromes</td>
</tr>
<tr>
<td>Cystic fibrosis</td>
<td>-K-ras</td>
</tr>
<tr>
<td>Previous partial gastrectomy</td>
<td>-p53</td>
</tr>
<tr>
<td>Blood group A</td>
<td></td>
</tr>
<tr>
<td>Familial and genetic factors</td>
<td></td>
</tr>
</tbody>
</table>

2.3. Classification of tumors of the liver, gallbladder and pancreas

The histological WHO classification of liver tumors (WHO 1978) and its revised version (WHO 1994) divide the tumors into eight main groups (epithelial, nonepithelial, miscellaneous, unclassified, hemopoietic and lymphoid, metastatic tumors, epithelial abnormalities and tumor-like lesions) that are also subdivided into smaller ones. HCC arising from the hepatocytes belongs to the malignant subgroup under the title epithelial tumors. More than 90% of all primary liver cancers are HCCs (Crawford 1999). Tumors can be divided histologically into grades I to IV based on their differentiation (Edmonson & Steiner 1954).

The WHO classification of tumors of the gallbladder divide tumors into seven main groups (epithelial, endocrine, non-epithelial, miscellaneous, unclassified, secondary and tumor-like lesions) and these are again classified into several subgroups. Epithelial
tumors include benign tumors, dysplasias and malignant tumors, e.g. adenocarcinomas (WHO 1991). The subcategory of dysplasias (mild, moderate and severe) precede to carcinoma in situ when the degree of atypia increases and eventually leads to invasive carcinoma of the gallbladder. It can be graded in grades I to III. Most of the malignant tumors of the gallbladder are adenocarcinomas showing varying degrees of differentiation (Rosai 1996a).

Pancreatic tumors are histologically classified according to WHO into nine main categories (epithelial tumors, tumors of pancreatic islets, non-epithelial, miscellaneous, unclassified tumors, hemopoietic and lymphoid neoplasms, metastatic tumors, epithelial abnormalities and tumor-like lesions) with several subgroups. Malignant epithelial tumors contain adenocarcinoma, squamous cell carcinoma, cystadenocarcinoma, acinar cell carcinoma and undifferentiated carcinoma (WHO 1978). Ductal adenocarcinoma of the exocrine pancreas and its variants comprise 85% of all cases of pancreatic malignancy (Rosai 1996b). Ductal adenocarcinomas are graded histologically in grades I to III based on their glandular differentiation, mucin production, mitoses (per 10 HPF, grade I < five to grade III > ten) and nuclear atypia. Tumor grade increases simultaneously with loss of glandular differentiation, growth of mitotic count, decreasing mucin production and increasing nuclear atypia (Klöppel et al. 1985).

The TNM staging for a cancer is determined according to its anatomic extent at the time of diagnosis. The TNM classifications of the tumors of the liver, gallbladder and pancreas are presented in Tables 4, 5 and 6.

Table 4. TNM classification of tumors of the liver. Sobin & Wittekind 1997.

<table>
<thead>
<tr>
<th>Category</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>TX</td>
<td>Primary tumor cannot be assessed</td>
</tr>
<tr>
<td>T0</td>
<td>No evidence of primary tumor</td>
</tr>
<tr>
<td>T1</td>
<td>Solitary tumor 2 cm or less in greatest dimension without vascular invasion</td>
</tr>
<tr>
<td>T2</td>
<td>Solitary tumor 2 cm or less in greatest dimension with vascular invasion or multiple tumors limited to one lobe, none more than 2 cm in greatest dimension without vascular invasion or solitary tumor more than 2 cm in greatest dimension without vascular invasion</td>
</tr>
<tr>
<td>T3</td>
<td>Solitary tumor more than 2 cm in greatest dimension with vascular invasion or multiple tumors limited to one lobe, none more than 2 cm in greatest dimension with vascular invasion or multiple tumors limited to one lobe, any more than 2 cm in greatest dimension with or without vascular invasion</td>
</tr>
<tr>
<td>T4</td>
<td>Multiple tumors in more than one lobe or tumor(s) involve(s) a major branch of the portal or hepatic vein(s); or tumor(s) with direct invasion of adjacent organs other than gallbladder; or tumor(s) with perforation of visceral peritoneum</td>
</tr>
<tr>
<td>NX</td>
<td>Regional lymph nodes cannot be assessed</td>
</tr>
<tr>
<td>N0</td>
<td>No regional lymph node metastasis</td>
</tr>
<tr>
<td>N1</td>
<td>Regional lymph node metastasis</td>
</tr>
<tr>
<td>MX</td>
<td>Presence of distant metastasis cannot be assessed</td>
</tr>
<tr>
<td>M0</td>
<td>No distant metastasis</td>
</tr>
<tr>
<td>M1</td>
<td>Distant metastasis</td>
</tr>
</tbody>
</table>

T=tumor, N=nodus, M=metastasis
Table 5. TNM classification of tumors of the gallbladder. Sobin & Wittekind 1997.

<table>
<thead>
<tr>
<th>Category</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>TX</td>
<td>Primary tumor cannot be assessed</td>
</tr>
<tr>
<td>T0</td>
<td>No evidence of primary tumor</td>
</tr>
<tr>
<td>Tis</td>
<td>Carcinoma in situ</td>
</tr>
<tr>
<td>T1</td>
<td>Tumor invades lamina propria (T1a) or muscle layer (T1b)</td>
</tr>
<tr>
<td>T2</td>
<td>Tumor invades perimuscular connective tissue, no extension beyond serosa or into liver</td>
</tr>
<tr>
<td>T3</td>
<td>Tumor perforates serosa (visceral peritoneum) or directly invades into one adjacent organ or both (extension 2 cm or less into liver)</td>
</tr>
<tr>
<td>T4</td>
<td>Tumor extends more than 2 cm into liver and/or into two or more adjacent organs (stomach, duodenum, colon, liver, pancreas, omentum, extrahepatic bile ducts, any involvement of liver)</td>
</tr>
<tr>
<td>NX</td>
<td>Regional lymph nodes cannot be assessed</td>
</tr>
<tr>
<td>N0</td>
<td>No regional lymph node metastasis</td>
</tr>
<tr>
<td>N1</td>
<td>Metastasis in cystic duct, pericoledochal, and/or hilar lymph nodes (i.e. in the hepatoduodenal ligament)</td>
</tr>
<tr>
<td>N2</td>
<td>Metastasis in peripancreatic (head only), periduodenal, periportal, coeliac and/or superior mesenteric lymph nodes</td>
</tr>
<tr>
<td>M</td>
<td>See Table 4.</td>
</tr>
</tbody>
</table>


<table>
<thead>
<tr>
<th>Category</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>TX</td>
<td>Primary tumor cannot be assessed</td>
</tr>
<tr>
<td>T0</td>
<td>No evidence of primary tumor</td>
</tr>
<tr>
<td>Tis</td>
<td>Carcinoma in situ</td>
</tr>
<tr>
<td>T1</td>
<td>Tumor limited to the pancreas, 2 cm or less in greatest dimension</td>
</tr>
<tr>
<td>T2</td>
<td>Tumor limited to the pancreas, more than 2 cm in greatest dimension</td>
</tr>
<tr>
<td>T3</td>
<td>Tumor extends directly into any of the following: duodenum, bile duct, peripancreatic tissues</td>
</tr>
<tr>
<td></td>
<td>Tumor extends directly into any of the following: stomach, spleen, colon, adjacent large vessels</td>
</tr>
<tr>
<td>NX</td>
<td>Regional lymph nodes cannot be assessed</td>
</tr>
<tr>
<td>N0</td>
<td>No regional lymph node metastasis</td>
</tr>
<tr>
<td>N1</td>
<td>Regional lymph node metastasis</td>
</tr>
<tr>
<td>N1a</td>
<td>Metastasis in a single regional lymph node</td>
</tr>
<tr>
<td>N1b</td>
<td>Metastasis in multiple regional lymph node</td>
</tr>
<tr>
<td>M</td>
<td>See Table 4.</td>
</tr>
</tbody>
</table>
2.4. Apoptosis

2.4.1. History

In 1965 John Kerr, an Australian pathologist, recognized that there were two morphologically distinct types of cell death, necrosis and another one causing a cell to shrink and become denser. He named this phenomenon shrinkage necrosis. Almost a decade later Kerr et al. 1972 introduced the term apoptosis for an active, morphologically distinctive, inherently programmed phenomenon involved e.g. in normal embryonic development and malignant neoplasms. The term apoptosis was previously used to describe the dropping of the leaves from trees in the autumn and it means literally “falling off” or “dropping off”, (apó = from, ptósis = a fall) (reviewed by Majno & Joris 1995 and Cummings et al. 1997). Since then, knowledge about apoptosis has increased and particularly during the past few years its basic molecular mechanisms have been revealed. Still there are many unanswered questions and that is why apoptosis is one of the most extensively studied fields in biomedical research.

2.4.2. Morphology of apoptosis and necrosis

Apoptosis is a biochemically regulated cell death program defined by characteristic morphological and biochemical changes. Necrosis is associated with a destruction of a group of cells in the same area, whereas apoptosis involves only scattered single cells (Kerr et al. 1994). The earliest recognizable morphological changes of apoptosis are compaction and segregation of the nuclear chromatin to sharply delineated granular masses along the nuclear envelope. Simultaneously with the nuclear changes, the dying cell separates from its neighbors, the cytoplasm condenses, cell volume decreases and the convolution of nuclear and cellular outline is usually evident. The nucleus splits into discrete fragments. In the end, cells form extensive surface blebs and protuberances to produce membrane-bound apoptotic bodies with well-preserved cytoplasmic organelles. Apoptotic bodies are swiftly phagocytosed by adjacent macrophages (Kerr et al. 1972, Kerr et al. 1994, Cummings et al. 1997). The morphologically visible process of apoptosis takes a few hours, the majority of the time is spent to undergo the degradation within the phagocytic cell (Wyllie 1997b). All this is in contrast with the features of necrosis, which includes the swelling of the dying cell because of membrane damage, and demolishing of the cytosolic structures, which leads to a release of lysosomal enzymes and causes an inflammatory reaction in the neighboring cells (Kerr et al. 1994, Wyllie 1997b). During apoptosis the plasma membrane remains intact and there is no inflammation involved (Kerr et al. 1994).

Even though apoptosis and necrosis are morphologically separated, there is evidence that they may partly be commonly regulated (Leist & Nicotera 1997). When presented in high doses, certain stimuli can induce both apoptosis and necrosis and apoptosis-inducing stimuli can cause necrotic cell death in vivo (Leist & Nicotera 1997). Also many apoptosis-causing stimuli (e.g. heat shock, hypoxia, viruses, radiation, nitric oxide etc.)
may cause necrosis (Leist & Nicotera 1997). In case of tumors, the number of apoptotic cells is increased around the necrotic areas (Arai & Katayama 1997). If the concentration of endogenous caspases in the cytosol is low, the cell reacts by necrosis; if it is high, the caspase cascade is preferentially activated, leading to apoptosis (Green & Reed 1998).

2.4.3. Apoptosis in physiological, developmental and noncarcinomatous pathological processes

Programmed cell death or apoptosis is designed to eliminate unwanted cells through activation of a controlled, internally programmed chain of events. It takes place in a variety of biologically significant situations. Among these is the programmed destruction of cells during embryogenesis including implantation, organogenesis, developmental involution and metamorphosis. For example, the loss of tail and the emerging of digits and toes from early hand or foot plate is a result of apoptotic death (Cummings et al. 1997, Saunders 1966). Also the peeling of sunburned skin results from apoptosis (Milligan & Schwartz 1997). Cell death during the development of the nervous system is apoptotic. Up to 50% of neurons die soon after forming synaptic connections with their target cells (Cummings et al. 1997, Raff et al. 1993). Hormone-dependent involution in the adult, such as endometriual cell breakdown during the menstrual cycle, the regression of the lactating breast after weaning, ovarian follicular atresia in the menopause and prostatic atrophy after castration occur also by apoptosis (Cummings et al. 1997, Hopwood & Levison 1975, Hurwitz & Adashi 1993, Kerr & Searle 1973, Walker et al. 1989). Cell deletion in proliferating cell populations, such as gastrointestinal epithelium takes place apoptotically (Cummings et al. 1997, Hall et al. 1994). Cell death induced by cytotoxic T-cells is responsible for recognizing and removing transformed and virally infected cells (Berke 1994, Cummings et al. 1997, Walker et al. 1988).

There is evidence that apoptosis plays a significant part in pathological processes such as stroke, myocardial infarction, heart failure and in chronic neurodegenerative conditions such as Alzheimer’s disease, Parkinson’s disease and ALS (Cotman & Anderson 1995, Olivetti et al. 1997, Wyllie 1997b, Saraste et al. 1997). Diseases associated with the induction or inhibition of programmed cell death are presented in Table 7.

<table>
<thead>
<tr>
<th>Association with inhibition of apoptosis</th>
<th>Association with increased apoptosis</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cancer</td>
<td>AIDS</td>
</tr>
<tr>
<td>Autoimmune disorders</td>
<td>Neurodegenerative disorders</td>
</tr>
<tr>
<td>SLE</td>
<td>Alzheimer’s disease</td>
</tr>
<tr>
<td>Immune-mediated</td>
<td>Parkinson’s disease</td>
</tr>
<tr>
<td>glomerulonephritis</td>
<td>ALS</td>
</tr>
<tr>
<td></td>
<td>Retinitis pigmentosa</td>
</tr>
<tr>
<td></td>
<td>Cerebellar degeneration</td>
</tr>
<tr>
<td>Viral infections</td>
<td>Ischemic injury</td>
</tr>
<tr>
<td>Herpesviruses</td>
<td>Myocardial infarction</td>
</tr>
<tr>
<td>Poxviruses</td>
<td>Stroke</td>
</tr>
<tr>
<td>Adenoviruses</td>
<td>Reperfusion injury</td>
</tr>
<tr>
<td></td>
<td>Toxin induced liver disease</td>
</tr>
<tr>
<td></td>
<td>Alcohol</td>
</tr>
</tbody>
</table>

2.4.4. Apoptosis and carcinogenesis

A failure to activate apoptosis after DNA injury is one route to carcinogenesis (Wyllie 1997b). Oncogene activation and oncosuppressor deficiency may cancel the apoptosis of cells bearing DNA damage and allow them to start building a tumor (Lyons & Clarke 1997). The p53 gene product is required for the initiation of apoptosis in response to genotoxic damage (Thompson 1995). The failure of cells to undergo apoptosis in response to DNA damage may underlie the enhanced resistance to radiation and chemotherapeutic agents observed in tumors that are deficient in p53 (Lu & Lane 1993, Thompson 1995). Generally, apoptosis is increased in cancer, although cancer is usually classified as associated with inhibition of apoptosis as a primary dysfunction. For instance in follicular B-cell lymphoma, inhibition of apoptosis has been shown to play a decisive role in the development of neoplasia (Tsujimoto et al. 1985). Increased apoptosis in tumors may be due to the activation of cancer genes in the process of neoplastic development, accumulation of T cells launching a FAS-mediated apoptosis in tumor cells or loss of cell adhesion or hypoxia (reviewed by Soini et al. 1998a). c-myc and ras are proto-oncogenes that have a role in the apoptotic machinery. The proto-oncogene c-myc has a pivotal role in growth control, differentiation and apoptosis. When inadequately expressed it has been associated with neoplasms. It induces proliferation in the presence of growth factors and acts apoptotically when they are absent (Hoffmann & Liebermann 1998). Overexpression of ras may lead to either increased or decreased apoptosis (Kauffmann-Zeh et al. 1997, Trent et al. 1996, Ward et al. 1997).
2.4.5. Programmed cell death in the invertebrates

The genes and proteins involved in apoptosis have first been identified and characterized in the nematode *Caenorhabditis elegans*. During the development of the *C. elegans*, 131 of the 1090 somatic cells undergo programmed cell death. Its cell death pathway is in many ways a miniature version of the mammalian apoptotic pathway. Genetic studies have revealed at least 11 genes that define it (Hengartner & Horvitz 1994a). Three of these play crucial roles in regulating and executing the death sentence. Two of them, ced 3 and ced 4 are required for the initiation of cell death and a third one, ced 9, acts to protect the cells from dying (Hengartner & Horvitz 1994a,b). Ced 9 is a member of the bcl-2 family of apoptosis regulators, ced 4 is homologous to Apaf-1, which promotes caspase activation and ced 3 is similar to mammalian caspases (Hengartner & Horvitz 1994b, Yang & Korsmeyer 1996, Zou et al. 1997, Adams & Cory 1998, Thornberry &Lazebnik 1998). Ced 4 binds to ced 3 and promotes ced 3 activation, whereas ced 9 binds to ced 4 and prevents it from activating ced 3 (Chinnayan et al. 1997a, Spector et al. 1997, Seshagiri & Miller 1997, Chinnayan et al. 1997b). Normally, ced 9 is complexed with ced 4 and ced 3 forming an inactive complex, the apoptosome, and keeping ced 3 inactive. Apoptosis stimuli cause ced 9 dissociation, allowing ced 3 activation and thereby committing cells to die by apoptosis (reviewed by Hengartner 1998).

In the fruit fly, *Drosophila melanogaster*, large amounts of cells die during embryonic development and metamorphosis (Abrams et al. 1993, McCall &Steller 1997, Steller 1995, Truman et al. 1992). These deaths involve caspase activation and the characteristic morphology of apoptosis (McCall & Steller 1997). In *Drosophila*, molecular analysis has led to the isolation of three novel cell death genes, reaper (rpr), head involution defective (hid) and grim, all appearing to integrate different signals regulating apoptosis (Chen et al. 1996, Grether et al. 1995, White et al. 1994). Ectopic expression of either rpr, hid or grim induces apoptosis in cultured cells and in many different cell types in transgenic animals (Bergmann et al. 1998a, Chen et al. 1996, Grether et al. 1995, McNabb et al. 1997, Pronk et al. 1996, White et al. 1994, Zhou et al. 1997). While hid is also expressed in cells not undergoing apoptosis (Grether et al. 1995), rpr and grim genes appear to be expressed only in cells doomed to die (Bergmann et al. 1998a, Chen et al. 1996, White et al. 1994). An other important class of genes, mutations of which have been isolated in cell death screens are *Drosophila* homologs of inhibitor of apoptosis proteins (IAPs) (Hay et al. 1995). Several IAPs have been shown to inhibit apoptosis through direct binding to caspases and inhibition of their activation (Deveraux et al. 1997, Hay et al. 1995). rpr, hid and grim may trigger apoptosis by preventing IAPs from blocking caspase activation (Bergmann et al. 1998b).

2.4.6. Biochemical features of apoptosis

The apoptotic process can be subdivided into three different phases: initiation (cells obtain a stimulus triggering apoptosis), effector phase (apoptotic machinery is activated but still subject to regulation) and degradation (process cannot be regulated anymore)
(Kroemer et al. 1995, Thompson 1995, Susin et al. 1997). Rapid regular nuclear DNA fragmentation has been regarded as the biochemical hallmark of apoptosis (Cummings et al. 1997, Wyllie 1980). Apoptotic cells exhibit an initial cleavage of DNA into large 50 to 300 kilobase pair fragments (Cummings et al. 1997, Oberhammer et al. 1993, Wyllie 1997a). Apoptosis is characterized by double-strand cleavage at the linker regions between nucleosomes, producing a series of fragments that are multiples of 180 to 200 base pair lengths, which can be detected in DNA electrophoresis as a typical ladder pattern (Kerr et al. 1994, Wyllie 1980). Internucleosomal cleavage of DNA is considered to arise from the activation of endonucleases (Oberhammer et al. 1993).

2.4.7. Tumor suppressor gene p53

The tumor suppressor gene p53, known as a “guardian of the genome” participates in DNA repair following DNA damage (Lane 1992b). There are 11 exons in the p53 gene which encodes a 53 kDa nuclear phosphoprotein with 393 amino acids (reviewed by Soussi et al. 1990). In the event of cellular DNA damage, p53 accumulates in the nucleus, leading to a halt of the cell cycle at the G1-S boundary, thus enabling DNA repair to occur (Lane 1992a). If the repair fails, p53 triggers apoptosis in damaged cells (Lane 1992a, Younish-Rouach et al. 1991). Besides DNA damage due to cytotoxic drugs, free radicals or irradiation, triggers for p53 dependent apoptosis include e.g. oncogene activation (such as expression of mitogenic oncogene c-myc), genotoxicity, hypoxia, hyperoxia, heat shock, growth factors, virus infection and metabolic changes (reviewed by Prives & Hall 1999, Bennett 1999, Gottlieb & Oren 1996, Wagner et al. 1994). Dysfunction of p53 protein, e.g. due to a mutation, leads to an increased mutational frequency in the affected cell population and eventually to neoplastic transformation (Lane 1992b). The p53 pathway is presented in Figure 1. p53 promotes programmed cell death in a manner that involves downregulation of bcl-2 and upregulation of bax (Miyashita et al. 1994, Miyashita & Reed 1995, Bennett 1999), p53 inducing genes PIGs (Polyak et al. 1997, Gottlieb & Oren 1998) or Fas related pathways (Owen-Schaub et al. 1995, Bennet et al. 1998). Caspases participate in the regulation of p53 function through mdm2. The mdm2 oncogene can bind to the p53 protein and negatively regulate its functions in transcription, cell cycle arrest and apoptosis (Chen et al. 1997). Caspases cleave mdm2 allowing p53 to enter the nucleus and perform its function (Nicholson & Thornberry 1997).

2.4.8. Induction of apoptosis

The induction of apoptosis can be influenced by a variety of death-triggering signals (Thompson 1995) presented in Table 8. Both injury and physiological stimuli can activate common effector events through caspase activation (Wyllie 1997a). Injury to DNA, plasma membranes and mitochondria together with injury inflicted through injection of cytotoxic T-cell granules all activate terminal events, although in different ways (Wyllie 1997a). For instance, UV-radiation can cause double strand breaks and induction of apoptosis due to injury of DNA (Wyllie 1997b). Releasing of ceramide from membrane
lipids on digestion by sphingomyelinases is a signal for the induction of apoptosis (Haimovitz-Friedman et al. 1994, Basu & Kolesnick 1998). Diverse stresses such as ionizing and UV radiation, heat and chemotherapeutic agents activate the sphingomyelin pathway (Haimovitz-Friedman et al. 1994, Basu & Kolesnick 1998).


<table>
<thead>
<tr>
<th>Physiologic activators</th>
<th>Damage-related inducers</th>
<th>Therapy-associated agents</th>
<th>Toxins</th>
</tr>
</thead>
<tbody>
<tr>
<td>TNF family</td>
<td>Heat shock</td>
<td>Chemotherapeutic drugs</td>
<td>Ethanol</td>
</tr>
<tr>
<td>Transforming growth factor β</td>
<td>Viral infection</td>
<td>Gamma radiation</td>
<td></td>
</tr>
<tr>
<td>Neurotransmitters</td>
<td>Bacterial toxins</td>
<td>UV radiation</td>
<td></td>
</tr>
<tr>
<td>Growth factor withdrawal</td>
<td>Cytolytic T cells</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Loss of matrix attachment</td>
<td>Nutrient deprivation</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Calcium</td>
<td>Oncogenes</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Glucocorticoids</td>
<td>Tumor suppressors</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Oxidants</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Free radicals</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

2.4.8.1. The role of mitochondria in apoptosis

Recent studies have indicated that mitochondria are part of the central apoptotic pathway. Mitochondrial cytochrome c translocation to cytosol as a consequence of mitochondrial injury can trigger apoptosis (Bossy-Wetzel et al. 1998). The molecular mechanisms responsible for cytochrome c translocation from mitochondria to cytosol during apoptosis are for the most part unknown. There are, however, theories that have been proposed to explain the mechanisms. First, it may be mediated by opening of a multiprotein complex formed at the contact sites between the mitochondrial outer and inner membrane (Bossy-Wetzel & Green 1999). Second, release may be caused by mitochondrial swelling and outer membrane rupture without loss in inner membrane potential (Vander Heiden et al. 1997, Bossy-Wetzel & Green 1999). Third, it is possible that cytochrome c release may involve a specific channel in the outer membrane (Bossy-Wetzel & Green 1999). Finally, there may be a mechanism other than any of those described above responsible for the release. After being released into the cytosol cytochrome c binds to Apaf-1 and causes its oligomerization. Apaf-1 activates the cell death initiator caspase, pro-caspase 9 leading to the activation of caspase 9 and the cleavage and activation of downstream caspases (Bossy-Wetzel & Green 1999). Bcl-2 and bcl-Xl, localized in the mitochondrial outer membranes, prevent cytochrome c release and cell death (Kim et al. 1997, Kluck et al. 1997, Yang et al. 1997). On the other hand, recent evidence shows that specific cleavage of bcl-2 at Asp34 by caspase 3 promotes the release of cytochrome c from mitochondria (Kirsch et al. 1999). There are also data about the role of bax and bcl-2 in Fas-mediated
apoptosis which demonstrates, that bax membrane translocation occurs during apoptosis induced by ligation of Fas (Murphy et al. 1999). Translocation is specific to mitochondria and precedes a loss of mitochondrial respiratory function and cell membrane integrity. Insertion of bax to the mitochondrial membrane was strongly associated with release of cytochrome c from the mitochondria (Murphy et al. 1999).

AIF (apoptosis inducing factor) is another apoptosis-promoting agent released from the mitochondria. AIF is a caspase-independent death effector, which translocates to the nucleus and causes chromatin condensation and large-scale DNA fragmentation (Susin et al. 1999).

2.4.9. Bcl-2 family proteins

Bcl-2 related proteins play a pivotal role in determining whether a cell will live or die. Of the apoptosis-regulating proteins, bcl-2 has been most extensively studied in clinical tumor material. Bcl-2 was found to be involved in the t(14;18) chromosomal translocation and therefore frequently overexpressed in low-grade B cell lymphomas, preventing them from apoptosis (Tsujimoto et al. 1985). The bcl-2 family is a group of apoptosis-regulating genes which are able to inhibit or promote apoptosis (Reed 1994, Hockenbery 1994, White 1996, Yang & Korsmeyer 1996, Kroemer 1997). At the moment, the bcl-2 family includes at least 19 mammalian members listed in Table 9 (Gross et al. 1999).


<table>
<thead>
<tr>
<th>Anti-apoptotic members</th>
<th>Pro-apoptotic members</th>
<th>BH3-domain only</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bcl-2</td>
<td>Bax</td>
<td></td>
</tr>
<tr>
<td>Mcl-1</td>
<td>Bak</td>
<td></td>
</tr>
<tr>
<td>Bcl-W</td>
<td>Bok</td>
<td></td>
</tr>
<tr>
<td>Bcl-X₁</td>
<td>Bcl-X₅</td>
<td></td>
</tr>
<tr>
<td>A₁</td>
<td>Bid</td>
<td>*</td>
</tr>
<tr>
<td>NR-13</td>
<td>Bad</td>
<td>*</td>
</tr>
<tr>
<td>BOO</td>
<td>Bik</td>
<td>*</td>
</tr>
<tr>
<td></td>
<td>BIk</td>
<td>*</td>
</tr>
<tr>
<td></td>
<td>Hrk</td>
<td>*</td>
</tr>
<tr>
<td></td>
<td>Bim</td>
<td>*</td>
</tr>
<tr>
<td></td>
<td>NIP₃</td>
<td>*</td>
</tr>
<tr>
<td></td>
<td>NIX</td>
<td>*</td>
</tr>
</tbody>
</table>

Bcl-2 family members possess up to four conserved bcl-2 homology (BH) domains: BH₁, BH₂, BH₃ and BH₄ (reviewed by Gross et al. 1999). The BH₃ domain is the only one found in all bcl-2 family members and it serves as a critical death domain in the pro-apoptotic members. Many members also contain a carboxy-terminal hydrophobic domain, which is essential for targeting to membranes such as the mitochondrial outer membrane (Nguyen et al. 1993). There is a group of proapoptotic “BH3-domain only” proteins displaying sequence homology only within the BH3 domain (reviewed by Adams & Cory 1998). Pro-and antiapoptotic members of the bcl-2 family are able to homo-and heterodimerize depending on their relative concentration (Olsvai et al. 1993). The amount of apoptosis-inhibiting bcl-2/bax heterodimers versus apoptosis-promoting bax/bax homodimers determines cells susceptibility to survive or die (Yang & Korsmeyer 1996). When bax is in excess in cells, apoptosis is accelerated, on the other hand the overexpression of bcl-2 leads to heterodimerization with bax and repressed death (Yang & Korsmeyer 1996). Current data suggest that there are two potentially independent mechanisms for promoting cell death. One mechanism is based on dimerization and the other, an intrinsic, heterodimerization-independent function is probably related to the ability of these proteins to insert into membranes (Reed 1998). Although heterodimerization is not required for pro-survival function, opposite to early indications (Yin et al. 1994, Cheng et al. 1996, Kelekar et al. 1997), heterodimerization is essential for the pro-apoptotic activity in the BH3 domain group, but not for the bax group (Adams & Cory 1998).
2.4.10. Caspases

2.4.10.1. Structure and function of caspases

Caspases are a family of cysteine proteases representing the effector components of the apoptotic machinery. They cleave their target proteins specifically after aspartatic acid residues in a defined consensus sequence context (Barge et al. 1997, Harvey et al. 1997, Thornberry et al. 1994 and Patel et al. 1996). Caspases reside in the cytosol as inactive precursors composed of four distinct domains (prodomain, a large subunit, a small subunit and a linker region flanked by Asp residues) and need to be activated in order to perform their function (Thornberry et al. 1994, Harvey et al. 1997, Nuñez et al. 1998, Thornberry & Lazebnik 1998). Caspases participate in a cascade, see Figure 2, leading to fragmentation of the molecules into 10 and 20 kDa subunits, which heterodimerize and associate into tetramers which constitute the active enzyme. Caspases located upstream cleave and activate downstream caspases, which then cleave the various substrate proteins responsible for many biochemical and morphological events during apoptosis (Harvey et al. 1997, Thornberry et al. 1994 and Patel et al. 1996). Target proteins include for instance proteins of the DNA repairing system such as poly(ADP-ribose)-polymerase (PARP), many structural proteins, e.g. nuclear lamins, fodrin, β-katenin, cyto keratin 18, NuMA (nuclear matrix protein) and some oncoproteins like mdm 2 and tumor suppressor gene products such as retinoblastoma protein (Patel et al. 1996, Rao et al. 1996, Brancolini et al. 1997, Chen et al. 1997, Gueth-Hallonet et al. 1997, Tan et al. 1997, Hirata et al. 1998). Caspases are also able to activate DNAase, which leads to typical DNA fragmentation found in apoptosis (Enari et al. 1998, Jänicke et al. 1998). In addition caspases can regulate their own activation via positive and negative feedback and amplification; an active protease is able to activate its own precursor directly or indirectly, resulting in an exponential rate of activation (Thornberry & Lazebnik 1998).

To date, a family of at least 14 different caspases have been identified (Thornberry et al. 1997, Thronberry & Lazebnik 1998, Nuñez et al. 1998, Huppertz et al. 1999, Rudel 1999). Caspases have been divided into upstream (initiator) and downstream (effector) cell death proteases based on their sites of action in the proteolytic caspase cascade (reviewed by Nuñez et al. 1998). Initiators include caspases 2, 8, 9, 10, 11, 12 and 13, while caspases 3, 4, 5, 6 and 7 are effectors (Thornberry et al. 1997, Thornberry & Lazebnik 1998, Nuñez et al. 1998, Huppertz et al. 1999, Rudel 1999). They can also be divided into subgroups on the basis of their phylogenetic development, structure or their order in the caspase activation cascade (Alnemri et al. 1996, Barge et al. 1997, Harvey et al. 1997).
ACTIVATION SIGNAL $\rightarrow$ SENSOR $\rightarrow$ ADAPTER $\rightarrow$ INITIATOR PROCASPASE

$\downarrow$

ACTIVE INITIATOR

$\downarrow$

EFFECTOR PROCASPASE

$\downarrow$

ACTIVE EFFECTOR

$\downarrow$

APOPTOTIC SUBSTRATES

Fig. 2. Caspase activation mechanisms. Modified from Wolf & Green 1999.

2.4.10.2. Activation of the caspase cascade and the role of cytotoxic lymphocytes

Cytotoxic lymphocytes (CTL) participate in caspase cascade through a Ca$^{2+}$-independent mechanism which activates caspases directly from the cell membrane through ligand binding of the TNF-like (tumor necrosis factor) receptors such as APO1/FAS/CD95 and TNFR1 (Berke 1995, Dhein et al. 1995, Itoh et al. 1991, Ju et al. 1995, Muzio et al. 1996, Nagata 1997, Nagata & Golstein 1995, Suda et al. 1993, Wallach et al. 1997, Ashkenazi & Dixit 1998, Kidd 1998). Death receptors contain a cytoplasmic sequence, “death domain” (DD) coupling each receptor to caspase cascade (French & Tschopp 1999). Death domains can in turn associate with and activate the upstream caspases through death effector domain (DED) or caspase recruitment domain (CARD) interactions, leading to the activation of the downstream caspases and eventually apoptosis (Djerbi et al. 1999). The binding of Fas ligand to its receptor leads to oligomerization of the receptor and to formation of the death-inducing signaling complex (DISC), which includes the receptor, FADD/MORT (Fas associated death domain/mediator of receptor induced toxicity) and FLICE (FADD like ICE/procaspase 8) (Fraser & Evan 1996, Muzio et al. 1996, Nagata 1997). The result is oligomerization and activation of caspase 8 through self-cleavage, and activation cascade of other caspases,
such as caspase 3 and caspase 6 (Nicholson & Thornberry 1997, Hirata et al. 1998). Another CTL ligand, TNF, activates TNF-R1, which through an adapter molecule TRADD (TNF-R1-associated death domain) promotes activation of caspase 8 and apoptosis through FADD coupling or activation of survival signals as discussed in following chapter (Ashkenzi & Dixit 1998, Baker & Reddy 1998). Besides FADD, TNF-R1 can engage an adapter molecule RAIDD (RIP associated ICH-1/CED-3 homologous protein with a death domain) and through interaction with receptor interacting protein (RIP) it can activate caspase 2 (Baker & Reddy 1998, Varadachary & Salgame 1998). Fas ligand interactions activate caspase cascade and apoptosis also with a mechanism which involves activation of the JNK (c-Jun-N-terminal kinase) pathway mediated by Daxx (death domain associated protein) (Baker & Reddy 1998). The activation of caspase 10 is the result of the interaction between TRAIL (TNF-related apoptosis inducing ligand) and its receptors R1, R2 or R3 or TRAMP (TNF-receptor related apoptosis mediating protein) (Baker & Reddy 1998, Varadachary & Salgame 1998). The Ca\textsuperscript{2+}-dependent release of perforin and granzyme B from CTL granules is the other way for cytotoxic lymphocytes to cause apoptosis via the activation of caspase 3 and the caspase cascade downstream of it (Atkinson et al. 1998, Berke 1995, Froehlich et al. 1998).

Caspases can also be activated through substances such as cytochrome c or AIF released from the mitochondria (Kluck et al. 1997, Manon et al. 1997, Yang et al. 1997). Release of cytochrome c leads to activation of caspase 9 which in turn activates caspases 3, 6 and 7 (Li et al. 1997, Nuñez et al. 1998).

2.4.10.3. Inhibition of death receptor apoptosis

Death receptor apoptosis can be inhibited at the receptor level and at the effector stage (French & Tschopp 1999). Death receptor mediated apoptosis can be modulated by several antiapoptotic proteins such as FLICE- (FADD [Fas-associated death domain]-like IL-1β-converting enzyme) --inhibitory proteins (FLIPs) (Djerbi et al. 1999). Viral FLICE (vFLIPs) found in several herpesviruses and their cellular homologues (cFLIPs) constitute a novel class of inhibitors of death receptor-mediated apoptosis that prevent the association of upstream caspases 8 and 10 with the adapter molecule FADD through DED-DED interactions (Djerbi et al. 1999, French & Tschopp 1999). cFLIP expression has been shown to be overexpressed in human melanomas (Medema et al. 1999, French & Tschopp 1999). TNF ligand can bind to TNF-R1 and interact with adapter molecule TRADD leading to NF-κB (nuclear factor-kappaB) activation and survival signal (Baker & Reddy 1998). TNF-R1–mediated induction of cell survival appears to involve a second receptor-associated protein, RIP, which interacts with TRADD via its death domain (Baker & Reddy 1998). The overexpression of RIP induces NF-κB as well as JNK activation (Baker & Reddy 1998).

Cytokine response modifier A (Crm A) and p35 from baculovirus can prevent apoptosis by serving as non-cleavable substrates for ICE-related caspases (Varadachary & Salgame 1998). The inhibitor of apoptosis proteins (IAP) are a family of apoptotic inhibitors, first identified in the baculoviruses (LaCasse et al. 1998). It appears that the majority of IAPs inhibit apoptosis through direct caspase and pro-caspase inhibition,
primarily caspases 3 and 7 (Deveraux et al. 1997, Roy et al. 1997). Survivin, the smallest IAP cloned to date, is normally expressed in fetus, but not in adult differentiated tissue (Adida et al. 1998, Ambrosini et al. 1997). The strongest evidence for IAP involvement in cancer is found in survivin, which is expressed in several malignancies, e.g. in lung, colon, breast, prostate and pancreatic carcinomas (LaCasse et al. 1998).

2.4.11. Regulation of apoptosis

Although diverse signals are able to induce apoptosis in a wide variety of cell types, a number of genes regulating programmed cell death are conserved from worms to humans (Thompson 1995). Figure 3 represents a schematic model of mammalian cell death pathway, the events leading to caspase cascade and, eventually, to apoptosis.
Figure 3. Mammalian cell death pathway.
2.5. P-glycoprotein

The term “multidrug resistance” refers to a special form of clinical resistance of various tumors against a wide range of chemotherapeutic agents (Szakacs et al. 1998, Ramachandran et al. 1999). The definition of MDR includes resistance due to drug pumps and due to interference with apoptosis (Borst 1999). Many anticancer drugs act by inducing apoptosis. Decreased apoptosis can therefore lead to drug resistance, at least in cultured cells (Borst 1999). Multidrug resistance is caused by the overexpression of two membrane proteins, MDR1-Pgp and/or MRP (multidrug resistance protein) and it is a major obstacle in the chemotherapy of cancer (Szakacs et al. 1998, Ramachandran et al. 1999).

P-glycoprotein (Pgp) is a 170 kDa, membrane-bound glycoprotein encoded by the multidrug resistance (MDR1) gene (Dietel 1991, Gottesmann 1993, Itsubo et al. 1994, De Angelis et al. 1995). It functions as an ATP dependent pump transporting drugs and cytostatics out of the cytoplasm (Dietel 1991, Gottesmann 1993, Itsubo et al. 1994, De Angelis et al. 1995) and as a chloride channel (De Angelis et al. 1995). Pgp is expressed in many human cancers and normal tissues (Kaye 1998). mRNA of MDR1 and its product, Pgp (also called the multidrug transporter), are found at substantial levels in normal colon, small intestine, kidney, liver and adrenal gland as well as in capillaries of the brain and testis (Chin et al. 1992). Pgp expression has been found in tumors derived from tissues which normally express the MDR1 gene, such as carcinomas of the liver, colon, kidney and pancreas (Gottesmann 1993). In addition Pgp has been shown to correlate with a lack of chemotherapeutic response and a shorter disease free survival, e.g. in acute leukemia, neuroblastoma, ovarian cancer and breast carcinomas (Chin et al. 1993, Sinicrope et al. 1994). Tumors that develop from tissues normally expressing intermediate to high MDR1 gene levels are all intrinsically drug resistant (Charpin et al. 1994). It appears that MDR1 expression is an inherent characteristic of tumor cells, but the multidrug-resistant phenotype can also be acquired as a consequence of the chemotherapeutic agents (Charpin et al. 1994). In breast cancer expression of Pgp has been associated with p53 expression and cell proliferation (Charpin et al. 1994).
3. Aims of the present study

Hepatocellular, gallbladder and pancreatic carcinomas have extremely poor prognosis. The survival rates for these malignancies are low. During the past few years studies on programmed cell death, apoptosis, have been launched. Tumor growth is a sum of cell proliferation and cell loss. To clarify the biology of these highly mortal neoplasms, it is essential to investigate the occurrence and extent of apoptosis and its association with the growth and progression of cancer. The specific aims of the study were:

1. to determine the extent of apoptosis in HCC, gallbladder and pancreatic carcinoma (I, III, IV, V)

2. to study the expression of apoptosis-regulating proteins bcl-2, mcl-1 and bax in relation to the extent of apoptosis in these three tumor groups (I, III, IV, V)

3. to evaluate the expression of p53 and its association to apoptosis (I, II, unpublished data)

4. to evaluate P-glycoprotein as a prognostic marker in HCC and its association to the extent of apoptosis (II, unpublished data)

5. to evaluate survival in relation to apoptosis and other factors, such as caspases and bcl-2 family proteins (I-V), and to evaluate the growth index as and indicator of prognosis in HCCs and pancreatic carcinomas (I, unpublished data)
4. Materials and methods

4.1. Tumor material

The material for all studies had been fixed in 10% neutral formalin and embedded in paraffin. Representative samples were collected and re-evaluated for histopathological diagnosis and classified based on a light microscopical examination using conventional hematoxylin-eosin stained slides according to the criteria of the World Health Organization (WHO 1978, WHO 1991, WHO 1996). Description of the material used in each study is represented in Table 10.

4.2. 3′-end labeling of DNA in apoptotic cells

In order to detect apoptotic cells, in situ labeling of the 3′-ends of the DNA fragments generated by apoptosis-associated endonuclease was performed using the ApopTag in situ detection kit (Oncor, Gaithersburg, MD, USA) following the instructions laid out by manufacturer, with a few modifications. After dewaxing in xylene and rehydration in ethanol, the sections were incubated with 20 μg/ml Proteinase K (Boehringer Mannheim GmbH, Mannheim, Germany) at room temperature for 15 minutes. The endogenous peroxidase activity was blocked by incubating the slides in 2% hydrogen peroxide in phosphate buffered saline (PBS), pH 7.2. Terminal transferase enzyme was used to catalyze the addition of digoxigenin-labeled nucleotides to the 3′-OH ends of the fragmented DNA. The reaction was stopped with a buffer provided by the manufacturer after 60 minutes, after which anti-digoxigenin-peroxidase solution was applied on the specimens. The color was developed with diaminobenzidine-hydrogen peroxide, and the slides were thereafter lightly counterstained with hematoxylin. For control purposes tissue sections from hyperplastic lymph nodes were used.
Table 10. The material used in studies I-V.

<table>
<thead>
<tr>
<th>Article/Anatomic location</th>
<th>Diagnosis (number of cases)</th>
<th>Grade (no)</th>
</tr>
</thead>
<tbody>
<tr>
<td>I/ Liver</td>
<td>Hepatocellular carcinoma (33)</td>
<td>Grade I (2)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Grade II (15)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Grade III (15)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Grade IV (1)</td>
</tr>
<tr>
<td>II/ Liver</td>
<td>Hepatocellular carcinoma (31)</td>
<td>Grade I (2)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Grade II (14)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Grade III (15)</td>
</tr>
<tr>
<td>III/ Gallbladder</td>
<td>Adenocarcinoma (39)</td>
<td>Grade I (13)</td>
</tr>
<tr>
<td></td>
<td>Dysplasias (7)</td>
<td>Grade II (13)</td>
</tr>
<tr>
<td></td>
<td>Mild (1)</td>
<td>Grade III (13)</td>
</tr>
<tr>
<td></td>
<td>Moderate (3)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Severe (3)</td>
<td></td>
</tr>
<tr>
<td>IV/ Pancreas</td>
<td>Adenocarcinoma (44)</td>
<td>Grade I (14)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Grade II (24)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Grade III (6)</td>
</tr>
<tr>
<td>V/ Pancreas</td>
<td>Ductal adenocarcinoma (78)</td>
<td>Grade I (27)</td>
</tr>
<tr>
<td></td>
<td>Acinar carcinoma (3)*</td>
<td>Grade II (41)</td>
</tr>
<tr>
<td></td>
<td>Neuroendocrine carcinoma (3)*</td>
<td>Grade III (10)</td>
</tr>
<tr>
<td></td>
<td>Mucinous cystadenocarcinoma (2)*</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Squamous cell carcinoma (1)*</td>
<td>* = was not graded</td>
</tr>
<tr>
<td></td>
<td></td>
<td>due to the number of</td>
</tr>
<tr>
<td></td>
<td></td>
<td>cases</td>
</tr>
</tbody>
</table>

4.2.1. Assessment of the apoptotic index

Cells were defined as apoptotic if the whole nuclear area of the cell labeled positively and if it fulfilled the morphological criteria of apoptosis, such as the condensation of the nucleus and cell shrinkage (Kerr et al., 1972, Kerr et al., 1994). Apoptotic bodies were defined as small positively-labeled globular bodies in the cytoplasm of the tumor cells, which could be found either singly or in groups. Apoptotic bodies occurring in distinct groups, and likely to be originated from the same apoptotic cell, were recorded as one apoptotic event. The number of apoptotic cells and bodies was counted in ten high power fields (HPFs) and this figure was divided by the number of tumor cells in the same HPFs, and thus the percentage of apoptotic events per cell population was obtained. The apoptotic index was also estimated by light microscopy in a similar manner from hematoxylin-eosin stained slides in the same tumor sections in order to compare the results with those obtained by the TUNEL method. The microscope used in all evaluations was Olympus
System Microscope Model BHS-2 and the magnification used was 40x. The diameter of the field with this magnification is 400 μm. Apoptosis was not assessed in the vicinity of necrotic areas.

4.3. Immunohistochemical stainings

Sections (5μm) were cut from the specimens and placed on poly-l-lysine coated (Sigma Chemicals, St Louis, MO, USA) glass slides, air-dried overnight and stained within a few days. The sections (one representative section per case) were then dewaxed in xylene and rehydrated in graded alcohol. The endogenous peroxidase was consumed by immersing the sections in 0.1% hydrogen peroxide in absolute methanol for 20 minutes. Non-specific binding was blocked by incubating the slides in 20% fetal calf serum in phosphate-buffered saline (PBS) for 20 minutes. The proteins and their respective antibodies and incubation times used in these studies are listed in Table 11. After dewaxing and rehydration the antibodies requiring an antigen retrieval step were taken to microwave oven for three to ten minutes in citrate buffer (pH 6). All specimens were overlaid with the suitable secondary antibodies. For all stainings, the avidin-biotin-complex kit (ABC-kit, Dako A/S, Glostrup, Denmark) was then used according to the manufacturer’s instructions. In all cases sections were rinsed stringently, with several changes of PBS between each stage. For all the immunostainings, the color was developed by diaminobenzidine and the sections were lightly counterstained with hematoxylin or ethyl green and mounted with Eukitt (Kindler, Freiburg, Germany). Negative control stainings were carried out by substituting non-immune mouse, goat or rabbit serum for the primary antibodies. For bcl-2, bax, mcl-1, caspases 3, 6 and 8 and PCNA immunostaining, a lymph node with follicular hyperplasia or a breast carcinoma was used as a positive control. Positive control for p53 immunostaining was a lung carcinoma, previously shown to be strongly positive for p53 (Soini et al. 1992). Normal liver served as an internal positive control for P-glycoprotein immunostaining.

Evaluation of all the stainings was performed by two persons independently and a consensus was formed on cases where the opinions differed. A combined score, based on both intensity and the quantity of the immunostaining, was composed by adding both the qualitative and quantitative scores, and then forming three main groups for the immunostainings of bcl-2, mcl-1, bax and caspases 3, 6, and 8 for the statistical analysis (III, V). For p53, PCNA and P-glycoprotein immunostainings, the percentage of positively stained tumor cells was evaluated in each section (IV, II). For bcl-2 and p53 stainings the results were classified as positive or negative and PCNA was again evaluated as the percentage of positively stained tumor cells (I).

4.4. Estimation of the mitotic count and necrosis

Apart from staining of PCNA, cell proliferation was also assessed by counting the number of mitotic figures per ten HPFs. The estimation was performed from the same
slides as the apoptotic index. The extent of necrosis was assessed light microscopically by evaluating the proportion of necrotic areas in the whole tumor tissue. The percentage of necrosis was the sum proportion of necrotic area/all tumor area. The estimation of necrosis was performed on all available tumor tissue slides.

4.5. Statistical analysis

The statistical analysis was performed with the SPSS for Windows program package (Chicago, IL, USA). Comparisons between groups were made using the two-tailed Student’s t-test. The significance of associations was determined using Fisher’s exact probability test and Spearman’s correlation analysis. The survival data and disease free interval were analysed according to the Kaplan-Meier method. The difference between survival and disease-free interval in different groups was analysed using the log rank, Breslow and Tarone-Ware tests. Probability values of less than 0.05 were considered significant.

Table 11. Antigens and their respective antibodies. See articles I to V for details.

<table>
<thead>
<tr>
<th>Antigen</th>
<th>Antibody</th>
<th>Cl</th>
<th>T</th>
<th>Dilution</th>
<th>Staining pattern</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bax</td>
<td>Bax ab</td>
<td>PC</td>
<td>+</td>
<td>1:1000</td>
<td>Cytoplasmic</td>
<td>Pharmingen</td>
</tr>
<tr>
<td>Bcl-2</td>
<td>Bcl-2 ab</td>
<td>MC</td>
<td>+</td>
<td>1:50</td>
<td>Cytoplasmic</td>
<td>Dako A/S</td>
</tr>
<tr>
<td></td>
<td>(clone 124)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Caspase-3</td>
<td>CPP32</td>
<td>PC</td>
<td>+</td>
<td>1:500</td>
<td>Cytoplasmic</td>
<td>Pharmingen</td>
</tr>
<tr>
<td>Caspase-6</td>
<td>Mch2p20</td>
<td>PC</td>
<td>+</td>
<td>1:100</td>
<td>Cytoplasmic</td>
<td>Santa Cruz Biotechnology Inc.</td>
</tr>
<tr>
<td>Caspase-8</td>
<td>Mch5p20</td>
<td>PC</td>
<td>+</td>
<td>1:100</td>
<td>Cytoplasmic</td>
<td>Santa Cruz Biotechnology Inc.</td>
</tr>
<tr>
<td>Mcl-1</td>
<td>Mcl-1 ab</td>
<td>PC</td>
<td>+</td>
<td>1:750/</td>
<td>Cytoplasmic</td>
<td>Pharmingen</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>1:1000</td>
<td></td>
<td></td>
</tr>
<tr>
<td>p53</td>
<td>CM-1</td>
<td>PC</td>
<td>–</td>
<td>1:1000</td>
<td>Nuclear</td>
<td>Novocastra Laboratories</td>
</tr>
<tr>
<td>PCNA</td>
<td>PCNA ab</td>
<td>MC</td>
<td>+</td>
<td>1:50</td>
<td>Nuclear</td>
<td>Dako A/S</td>
</tr>
<tr>
<td></td>
<td>(clone PC10)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>P-glycoprotein</td>
<td>C219</td>
<td>MC</td>
<td>+</td>
<td>1:20</td>
<td>Cell membrane</td>
<td>Signet Laboratories</td>
</tr>
</tbody>
</table>

Cl=clonality, PC=polyclonal, MC=monoclonal, T=pretreatment; +=microwave in citrate buffer, —=none
Pharmingen, San Diego, CA, USA; Dako A/S, Glostrup, Denmark; Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA; Novocastra Laboratories, Newcastle upon Tyne, UK; Signet Laboratories, Dedha, Massachusetts, USA.
5. Results

5.1. Apoptosis in HCC, gallbladder and pancreatic carcinomas

5.1.1. The extent of apoptosis in HCC (I)

The average apoptotic index in HCC was 0.73% and the average apoptotic index in non-neoplastic liver cells adjacent to the tumors was 0.10%. There were more apoptotic cells and bodies in the immediate proximity of the tumors and the frequency of apoptotic cells and bodies correlated significantly with each other (r=0.875, P<0.001). When proliferation, apoptosis or necrosis were considered separately, no statistically significant association with the survival time or post-operative disease-free interval was noticed. There was no significant association between apoptosis and necrosis, or between apoptosis and cell proliferation as determined by PCNA immunohistochemistry.

5.1.2. The extent of apoptosis in gallbladder carcinoma (III)

The average apoptotic index in gallbladder carcinomas and dysplasias was 0.68% as determined by the TUNEL method, and 0.59% according to the evaluation by light microscopy without 3'-end labeling. There was a statistically significant correlation between the apoptotic index as determined by the TUNEL method and evaluation by light microscopy without labeling (r=0.3456, P=0.022). There was a significantly lower extent of apoptosis in dysplasias and grade I tumors than in grade II-III tumors (P=0.003). The apoptotic indices in grade I, II and III carcinomas and in dysplasias are shown in Table 12. The apoptotic index also correlated with necrosis (r=0.4378, P=0.002); see Table 12. The extent of apoptosis was lower in dysplasias and T1-T2 tumors than in T3-T4 tumors (P=0.05). No significant association was found between patient survival and apoptosis in carcinomas, even though patients with tumors of a higher extent of apoptosis tended to have a worse outcome (P=0.16).
Table 12. Extent of apoptosis determined by the TUNEL method and by light microscopy without 3'-end labeling and percentage of necrosis in gallbladder dysplasias and carcinomas.

<table>
<thead>
<tr>
<th>Lesion</th>
<th>Apoptosis by TUNEL</th>
<th>Apoptosis without labeling</th>
<th>Necrosis (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>0.02</td>
<td>0.02</td>
<td>0.00</td>
</tr>
<tr>
<td>Dysplasia</td>
<td>0.06</td>
<td>0.14</td>
<td>0.00</td>
</tr>
<tr>
<td>Grade I carcinoma</td>
<td>0.53</td>
<td>0.42</td>
<td>6.62</td>
</tr>
<tr>
<td>Grade II carcinoma</td>
<td>0.64</td>
<td>0.67</td>
<td>2.14</td>
</tr>
<tr>
<td>Grade III carcinoma</td>
<td>1.34</td>
<td>0.98</td>
<td>13.40</td>
</tr>
</tbody>
</table>

5.1.3. The extent of apoptosis in pancreatic carcinoma (IV and V)

The mean apoptotic index in pancreatic adenocarcinoma, as determined by the TUNEL method, was 0.69% (range 0.01%-2.99%). There was a strong positive correlation between the apoptotic index determined by the TUNEL method and by light microscopy without 3'-end labeling (r=0.744, P<0.001). In non-neoplastic exocrine pancreatic epithelial cells, the apoptotic index was 0.07% and was increased in association with inflammation. The number of apoptotic cells correlated strongly with the apoptotic bodies (r=0.805, P<0.001). Ductal carcinomas had a higher extent of apoptosis (0.72%) than other histological types of tumors (0.19%) (P=0.041). No statistically significant association was found between apoptosis and necrosis or between apoptosis and cell proliferation. Nor was any statistically significant correlation seen between tumor stage and the extent of apoptosis. Tumor size did not correlate with apoptosis. In a group of radically operated pancreatic carcinomas (n=44) no statistically significant difference was observed in survival or disease-free interval after operation between cases with high (≥0.59%) or low (<0.59%) apoptotic index (P=0.23, P=0.94, respectively).

5.1.4. Design of a growth index (I and unpublished data)

Since HCCs are solid, mostly consisting of tumor cells with only scant stromal tissue we designed a growth index for the tumors based on a simple equation (Steel 1977) where:

Tumor growth = cell production - cell loss.

The values for positive staining for PCNA as a measure for cell proliferation and apoptosis together with necrosis as a measure of cell death were projected on a semiquantitative scale, and a growth index was designed as follows:

Growth index = 2xPCNA - (apoptosis score + necrosis score).

In HCC cases having a positive growth index, the survival of the patient was significantly shorter than in those with zero or negative growth index (P=0.004, by log-rank, P=0.006, by Breslow, P=0.004, by Tarone-Ware). There were significantly more
relapses in HCC cases with a positive growth index than in cases with a zero or negative one (P=0.05).

The growth index in pancreatic adenocarcinoma was determined in a similar way as in HCC. The associations were tested with the cases originating from article IV, because the cases in this material represented patients with a similar treatment and a rather uniform stage. There were 33 cases from which nine had a low (≤0) growth index. In line with HCC, patients with a low growth index had a better survival than others, but the association did not quite reach statistical significance (P=0.09).

5.2. The expression of apoptosis regulating proteins

5.2.1. p53 immunohistochemistry

The p53 protein becomes detectable by immunohistochemistry in tumor cells as a consequence of abnormal accumulation which is due to mutational events in the p53 gene or inactivation (Iggo et al. 1990, Momand et al. 1992). Unstable wild-type p53 protein does not accumulate in the nuclei and due to its short half life it is not detectable by immunohistochemistry (Iggo et al. 1990).

5.2.1.1. p53 and its associations in HCC (I,II)

Positive nuclear p53 immunostaining was found in 23% of HCCs. In non-neoplastic hepatocytes no p53 staining was found. There were significantly more p53-positive cases in the group with a high PCNA positivity than in the group with low PCNA positivity (P=0.001). There were significantly more p53-positive cases with a positive growth index than with zero or a negative index (P=0.015). No significant difference in the patient survival between p53 positive and p53 negative cases was found.

5.2.1.2. p53 and its associations in gallbladder carcinoma (unpublished data)

Positive immunostaining for p53 was seen in 57% of gallbladder carcinomas. p53 expression did not associate with apoptosis detected by TUNEL (P=0.60) or histologically verified apoptosis (P=0.66), high proliferation (P=0.48), necrosis (P=0.99) or survival (P=0.08).
5.2.1.3. *p53 and its associations in pancreatic carcinoma (III)*

41% of pancreatic adenocarcinomas were p53-positive. No significant association was found between a high (>0.59%) or low (≤ 0.59%) extent of apoptosis and the p53 status of the tumors. Grade III tumors were significantly more often p53-positive than grade I to II tumors (P=0.05). There were significantly more p53-positive tumors with a high PCNA-index than with a low PCNA-index (P=0.04). Patients with a p53-positive tumor were significantly younger (58±9.5 years) than those with a p53-negative tumor (65.6±8.8 years). No statistically significant difference in survival between p53-positive and p53-negative cases was found.

5.2.2. *Bcl-2 family proteins*

5.2.2.1. *Bcl-2 protein in HCC (I)*

Positive immunostaining of bcl-2 was found in only 1/33 (3%) of HCCs. In non-neoplastic hepatocytes no bcl-2 expression was found. Bcl-2 expression was, however, found in small proliferating bile ducts in association with cirrhosis and portal inflammation.

5.2.2.2. *Bcl-2 family proteins in gallbladder carcinoma (III)*

In non-neoplastic epithelium no bcl-2 positivity was observed. On the contrary, relatively strong mcl-1 or bax expression was seen in the non-neoplastic epithelium, especially in the areas with inflammation. In the neoplastic lesions, bcl-2 expression was detected in 10% of carcinomas and in 14% of dysplasias. 87% of tumors and dysplasias showed mcl-1 and all bax expression. Strong bax immunoreactivity was seen in 56% of the tumors while strong mcl-1 expression was seen in only 13% of the cases. In dysplasias 43% showed strong mcl-1 or bax expression. No association was found between the extent of apoptosis and the expression of bcl-2, mcl-1 or bax in gallbladder carcinomas. Neither was there any association between tumor grade, necrosis, survival, the expression of caspases 3, 6 and 8 and the expression of the bcl-2 group of proteins.

5.2.2.3. *Bcl-2 family proteins in pancreatic carcinoma (V)*

Positive cytoplasmic immunoreactivity for bcl-2, mcl-1 and bax was seen in a proportion of ductal and pancreatic epithelial cells. The cells of the islands of Langerhans were negative for bcl-2. Occasional positive staining could be seen with bax in a majority of the cases. However, a major population of cells of the islands of Langerhans expressed
mcl-1, the staining being especially pronounced in areas with inflammation. 13% of pancreatic carcinomas showed bcl-2 positive tumor cells. Positive bax immunoreactivity was seen in all tumors. The immunoreactivity was weak in 30% of the tumors. Positive mcl-1 immunoreactivity was seen in 86% of the tumors. The proteins of the bcl-2 family did not correlate with each other, with tumor grade or TNM status of the patients. The apoptotic index in cases showing bcl-2 immunoreactivity was 0.35%, while in cases with no immunoreactivity it was 0.64% (P=0.0013). Cases showing strong bax immunoreactivity had an apoptotic index of 0.70%, while cases with weak expression had an average index of 0.34% (P=0.008). There was no statistically significant difference between apoptotic index and mcl-1 expression.

5.2.3. Caspases 3, 6 and 8

5.2.3.1. Caspases 3, 6 and 8 in HCC (unpublished data)

The immunoreactivity for caspase 3 was mainly diffuse cytoplasmic, and in two cases also nuclear caspase 3 positivity was observed. The immunostaining of caspase 3 revealed diffuse cytoplasmic positivity in 92% of the cases. In 15% it was strong. The immunoreactivity for caspase 6 and 8 was granular and fragmented cytoplasmic staining. In a few cases also diffuse cytoplasmic staining was seen. Positive staining for caspase 6 was seen in 92% of the cases, with 31% showing a strong reaction. The immunostaining pattern for caspase 8 was similar to caspase 6, and a few cases with diffuse cytoplasmic staining was seen. Positive reaction was found in 73% and a strong one in 19% of the caspase 8 stainings. Table 13. shows immunohistochemical expressions of caspases 3, 6 and 8 in HCC. Caspase 3 expression and apoptosis did not associate with each other. Immunostaining of caspases 6 and 8 showed more apoptosis associated with moderate or strong staining. However, the difference did not reach statistical significance. Strong caspase 3 immunoreactivity associated with strong caspase 8 immunoreactivity (P=0.002). No other associations between different caspases were found.

Table 13. Immunohistochemical expression of caspases 3, 6 and 8 in 26 cases of HCC.

<table>
<thead>
<tr>
<th>Immunoreactivity</th>
<th>Caspase 3</th>
<th>Caspase 6</th>
<th>Caspase 8</th>
</tr>
</thead>
<tbody>
<tr>
<td>Negative</td>
<td>2 (8%)</td>
<td>2 (8%)</td>
<td>7 (27%)</td>
</tr>
<tr>
<td>Weak</td>
<td>12 (46%)</td>
<td>6 (23%)</td>
<td>11 (42%)</td>
</tr>
<tr>
<td>Moderate</td>
<td>8 (31%)</td>
<td>10 (38%)</td>
<td>3 (12%)</td>
</tr>
<tr>
<td>Strong</td>
<td>4 (15%)</td>
<td>8 (31%)</td>
<td>5 (19%)</td>
</tr>
</tbody>
</table>
5.2.3.2. Caspases 3, 6 and 8 in gallbladder carcinoma (III)

The immunoreactivity for all caspases was mostly diffuse cytoplasmic, but with caspases 6 and 8 also granular cytoplasmic positivity was seen. In one case of gallbladder carcinoma also nuclear caspase 3 positivity was observed. Weak occasional positive caspase 3, 6 and 8 intracytoplasmic positivity was seen in the non-neoplastic gallbladder epithelium. Epithelium seemed to express caspases more strongly in the areas of inflammation. In neoplastic epithelium the caspase immunoreactivity increased progressively according to the severity of the lesion and it was strongest in grade II-III lesions (Table 14). We did not find any statistically significant association between no and weak or strong caspase 3, 6 and 8 immunoreactivity and apoptosis. Similarly, no association was found in relation to necrosis. On the other hand, there was a strong statistical association between the expression of different caspases. Thus, strong caspase 3 immunoreactivity associated with strong caspase 6 and 8 immunoreactivity (P=0.003 and P=0.02, respectively) and strong caspase 6 immunoreactivity associated with strong caspase 8 immunoreactivity (P=0.00006). There was no association between caspase expression and survival of the patients in gallbladder carcinoma. Neither was there any association between chemotherapy treatment and strong caspase immunoreactivity in the tumors.

Table 14. Expression of caspases 3, 6 and 8 in gallbladder epithelial dysplasias and carcinomas.

<table>
<thead>
<tr>
<th>Lesion</th>
<th>Caspase 3 (no/weak/strong)</th>
<th>Caspase 6 (no/weak/strong)</th>
<th>Caspase 8 (no/weak/strong)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal epithelium</td>
<td>5/1/1</td>
<td>7/0/0</td>
<td>6/1/0</td>
</tr>
<tr>
<td>Dysplasias</td>
<td>0/6/1</td>
<td>3/3/1</td>
<td>3/3/1</td>
</tr>
<tr>
<td>Grade I carcinoma</td>
<td>0/9/4</td>
<td>4/8/1</td>
<td>5/7/1</td>
</tr>
<tr>
<td>Grade II carcinoma</td>
<td>1/8/4</td>
<td>2/8/3</td>
<td>1/8/4</td>
</tr>
<tr>
<td>Grade III carcinoma</td>
<td>1/5/7</td>
<td>3/7/3</td>
<td>3/6/4</td>
</tr>
</tbody>
</table>

5.2.3.3. Caspases 3, 6 and 8 in pancreatic carcinoma (V)

In non-neoplastic pancreatic acinar or ductal cells, only weak intracytoplasmic staining for caspases 3, 6 or 8 was occasionally seen. Cells of the islands of Langerhans were negative for caspase 6, but weak positive staining for caspase 3 could be seen in a small subpopulation of cells. Intense cytoplasmic staining for caspase 8 could be seen in a majority of cells of the islands of Langerhans, especially in areas of inflammation. The distribution of caspase 3, 6 and 8 immunoreactivity is shown in Table 15. The immunostaining of caspase 3 revealed diffuse cytoplasmic positivity in 80% of the cases. In 34% the staining was strong. The immunoreactivity for caspase 6 differed from patterns seen for caspase 3. It appeared as a granular and diffuse positive staining in 80%
of the cases. The immunoreactivity was partly diffuse, intracytoplasmic, but many times also granular intensely stained fragments, reminiscent of apoptotic bodies in the TUNEL staining, were seen. Nuclear fragments in necrotic areas also stained positively for caspase 6. The immunostaining pattern for caspase 8 was mainly diffuse cytoplasmic, but occasionally membrane-associated staining was seen. Sometimes also granular staining was noticed. The immunostaining for caspase 8 was positive in 74% of the tumors. There was no statistically significant difference between apoptosis and cases showing positive caspase 3, 6 or 8 expression.

Table 15. Immunohistochemical expression of caspases 3, 6 and 8 in pancreatic carcinoma.

<table>
<thead>
<tr>
<th>Immunoreactivity</th>
<th>Caspase 3</th>
<th>Caspase 6</th>
<th>Caspase 8</th>
</tr>
</thead>
<tbody>
<tr>
<td>Negative</td>
<td>15 (20 %)</td>
<td>17 (20 %)</td>
<td>22 (26%)</td>
</tr>
<tr>
<td>Weak</td>
<td>35 (46 %)</td>
<td>39 (46 %)</td>
<td>49 (58 %)</td>
</tr>
<tr>
<td>Strong</td>
<td>26 (34 %)</td>
<td>29 (34 %)</td>
<td>14 (16 %)</td>
</tr>
<tr>
<td>Total</td>
<td>76</td>
<td>85</td>
<td>85</td>
</tr>
</tbody>
</table>

5.3. Tumor necrosis and cell proliferation

5.3.1. Tumor necrosis and cell proliferation in HCC (I)

The average percentage of necrosis was 17% (range 0-95%) and the average mitotic count was 11.7 per ten HPFs (range 0.5-50.5) in HCCs. 36% of the tumors had a high proliferation index (>50% of cells positive) as judged by PCNA immunostaining. PCNA-positive non-neoplastic hepatocytes were found in close proximity to the tumor areas, suggesting increased proliferation in these areas. There were significantly more cases with more than ten mitoses per ten HPFs in the group with a high percentage of PCNA positivity (>50% of cells positive) than in the group with a low PCNA positivity (<50% of cells positive) (P=0.03). Similarly, there was a positive correlation between the percentage of PCNA-positive cells and the frequency of mitoses (r=0.524, P<0.05). No significant association was found between tumor necrosis and apoptosis, or PCNA index and apoptosis. A growth index for the tumors was designed based on cell proliferation, necrosis and apoptosis as discussed before. There was no statistically significant association between TNM classification and growth index. In tumors with positive growth index, the relapsing tumor growth could be seen in ultrasonography after 10.0±7.40 months, whereas in other cases it appeared after 34.0±28.9 months. The growth rate of the tumor in the former group was 0.42±0.33 cm/month, whereas in the latter group it was 0.16±0.11 cm/month.
5.3.2. Tumor necrosis and cell proliferation in pancreatic carcinoma (IV)

Proliferation index was high (+++/+++++) in 59% of the tumors as judged by PCNA immunohistochemistry. No significant association was found between apoptosis and PCNA immunostaining. The PCNA index did not associate with other parameters studied, including TNM status, grade, size, mitotic count, survival or disease free interval. No significant association was found between necrosis and mitotic count or any other parameter.

5.4. The expression of P-glycoprotein in HCC (II)

P-glycoprotein was expressed in 65% of HCCs. Immunoreactivity was mainly confined to the cell membrane, but in some cases globular intracytoplasmic staining was also seen. Adjacent normal liver tissue was also stained positively. Some of the proliferating bile ductules were Pgp positive at their luminal surface. Tumor grade or stage was not significantly associated with positive immunostaining, although tumors of lower grade (P=0.19) and higher stage (P=0.13) tended to be more heavily stained. In HCCs, tumors showing Pgp positivity had a lower apoptotic index (0.48±0.64%) than tumors showing no positivity (1.22±1.74%), but the association did not reach a statistical significance (P=0.096). No association was found between tumor necrosis (P=0.54) or the mitotic count (P=0.83) and Pgp immunoreactivity. Pgp did not associate with cell proliferation, measured by positive PCNA immunoreactivity and mitotic count. p53 and Pgp expressions did not associate significantly with each other. Patients with Pgp positive tumors had a shorter survival time (mean (SD) 47.0 (±12.0) months, 95% confidence interval (CI) 23.4-70.6 months) than those with Pgp negative tumors (72.3 (±14.3) months, 95% CI 44.3-100.3 months), but the difference was not, however, statistically significant. The disease-free interval (no evidence of recurrence either clinically or on laboratory investigation) was significantly shorter in patients with Pgp positive (38.5 (±10.9) months, 95% CI 17.1-60.0 months) than in those with negative tumors (86.2 (±13.3) months, 95% CI 60.1-112.2 months) (P<0.05). Chemotherapy did not associate to better survival in Pgp positive patients. There was no association between Pgp expression and liver cirrhosis or long term medication for conditions other than HCC, such as late onset diabetes, high blood pressure, heart failure, bronchial asthma and others.
5.5. A comparison between all parameters studied

Table 16. demonstrates a comparison of parameters studied in HCC, gallbladder and pancreatic carcinoma.

Table 16. Parameters studied in HCC, gallbladder and pancreatic carcinoma.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>HCC</th>
<th>Gallbladder carcinoma</th>
<th>Pancreatic carcinoma</th>
</tr>
</thead>
<tbody>
<tr>
<td>Apoptotic index</td>
<td>0.73%</td>
<td>0.68%</td>
<td>0.69%</td>
</tr>
<tr>
<td>Bcl-2</td>
<td>3%</td>
<td>10%</td>
<td>13%</td>
</tr>
<tr>
<td>Bax</td>
<td>-</td>
<td>100%</td>
<td>100%</td>
</tr>
<tr>
<td>Mcl-1</td>
<td>-</td>
<td>87%</td>
<td>86%</td>
</tr>
<tr>
<td>Caspase 3</td>
<td>92%</td>
<td>95%</td>
<td>80%</td>
</tr>
<tr>
<td>Caspase 6</td>
<td>92%</td>
<td>77%</td>
<td>80%</td>
</tr>
<tr>
<td>Caspase 8</td>
<td>73%</td>
<td>77%</td>
<td>74%</td>
</tr>
<tr>
<td>p53</td>
<td>23%</td>
<td>57%</td>
<td>41%</td>
</tr>
<tr>
<td>P-glycoprotein</td>
<td>65%</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

- = was not studied
6. Discussion

6.1. 3’-end labeling in the detection of apoptosis in tumors

Characteristic morphological features of apoptosis, including condensation of chromatin and cytoplasm, fragmentation of the cell and formation of apoptotic bodies, are manifest even in routinely stained sections (Kerr et al. 1972, Cummings et al. 1997, Kerr et al. 1994). Detection of apoptotic cells and bodies by light microscopy can be made from hematoxylin-eosin stained tissue samples by their morphological appearance. It is, however, time-consuming, and positive identification of apoptotic bodies of only 2 μm or larger can be made (Aihara et al. 1994). A more refined technique, based on the labeling of the free ends of the fragmented DNA allowing accurate identification of single apoptotic cells and bodies as small as 0.5 μm, was first described by Wijsman et al. (1993). This technique can be divided into two variants: in situ nick translation and the TUNEL (terminal deoxytransferase-mediated dUTP nick-end labeling) method. Comparison has shown that the TUNEL is more sensitive (Migheli et al. 1994, Mundle et al. 1995, Saraste 1999). Sources for misinterpretation in the TUNEL method lie in tissue pretreatment, concentration of the terminal transferase enzyme, the type and concentration of the fixative, fixation time and tissue type and the thickness of the tissue section (reviewed by Soini et al. 1998a, Saraste 1999). There is also a possibility that cells other than apoptotic ones, due to DNA damage, autolysis, tissue drying and necrosis, can become labeled. Long duration of formalin fixation can produce false negatives (reviewed by Soini et al. 1998a). A combination of morphological method and DNA end labeling method (TUNEL) produces the most reliable results possible with current knowledge, as was done in the present study.
6.2. Extent of apoptosis in HCC, gallbladder and pancreatic carcinomas compared to other malignant tumors

This study was undertaken to investigate the extent of apoptosis and apoptosis-regulating proteins in HCC, gallbladder and pancreatic carcinomas. In histological tumor material, apoptotic index is used as a measure of the extent of apoptosis. Most often apoptotic index is defined as a percentage of apoptotic cells and bodies per all tumor cells (Törmänen et al. 1995). It is more reliable to count a cluster of apoptotic bodies likely originating from the same dying cell as one death event, because a dying cell breaks up into several apoptotic bodies and a score of apoptotic bodies does not equal a cell death score. There are no uniform criteria for measuring the extent of apoptosis in tumors, and even though apoptotic index is widely used, the results between different authors vary, even among same tumor type. These differences can be explained by different technical and methodological factors. Defective apoptosis allows damaged, mutated cells to grow. A primary dysfunction in cancer is the inhibition of apoptosis. For instance in follicular B-cell lymphomas, inhibition of apoptosis has been convincingly shown to play a decisive role in the development of neoplasia (Tsujimoto et al. 1985). Low apoptotic indices (less than 1%) have been reported e.g. in malignant salivary gland tumors (Soini et al. 1998c).

In this study a low apoptotic index, 0.73%, was shown also in HCC. In gallbladder carcinoma the average apoptotic index was at about the same level as in HCC, being 0.68%. The average apoptotic index in pancreatic carcinoma as determined by the TUNEL method was also low, 0.69%. The apoptotic index was higher in ductal adenocarcinomas than in other types of carcinoma, the former group having an apoptotic index of 0.72% and the latter 0.19%. The results thus show a similar frequency of apoptosis in tumors of these three sites. When compared to tumors of other sites the frequency of apoptosis is relatively low. High apoptotic indices have been reported e.g. in small cell lung carcinoma (Eerola et al. 1997), prostate carcinoma (Koivisto et al. 1997) and high grade lymphomas (Soini et al. 1998b). On the other hand, high grade lymphomas show a significantly higher extent of apoptosis than low grade lymphomas (Soini et al. 1998b). According to our results there is a progressive increase in the extent of apoptosis from dysplasias to high grade tumors of the gallbladder. The results are in line with observations from other epithelial tumors, such as breast and bronchial preneoplastic lesions and carcinomas which show a similar progression in apoptosis (Mustonen et al. 1997, Törmänen et al. 1999). This is probably due to cumulative changes in apoptosis-regulating cancer genes which are activated during tumor progression. Such apoptosis-regulating genes include p53, Rb, ras and c-myc, the expression of which may change during tumor progression (reviewed by Soini et al. 1998a). An attempt to restore the epithelium back to original might also be an explanation for the increase in apoptotic index in line with progression of tumor grade. In breast (Mustonen et al. 1997) and thyroid (Basolo et al. 1997) carcinomas more apoptosis is seen in tumors of a high grade. In HCCs and pancreatic carcinomas no association between apoptosis and grade of the tumor was found.
6.3. Apoptosis in relation to necrosis, prognosis and cell proliferation

In addition to tumor grade or stage, there was also a statistically significant association between apoptosis and necrosis in gallbladder carcinoma. Even though apoptosis and necrosis are morphologically separate, there is evidence that they are partly commonly regulated. It has been reported that stimuli causing apoptosis at low doses may cause necrosis when the doses are high (Leist & Nicotera 1997). Apoptosis-inducing stimuli, such as heat shock, hypoxia, viruses, radiation and nitric oxide may cause necrosis (Leist et al. 1997). Depletion of intracellular ATP in human T cells shifts cell death from apoptosis to necrosis (Leist et al. 1997). The reaction of the cell to a destructive stimulus depends on the concentration of endogenous caspases in the cytosol. When the concentration is low, necrotic cell death is activated, if it is high, the caspase cascade is preferentially activated leading to apoptosis (Green & Reed 1998). The statistical association between apoptosis and necrosis might thus reflect the close association between the mechanisms of these phenomena. On the other hand, also methodological aspects may have an impact: necrotic cells have been reported to be labeled by the TUNEL method (Grasl-Kraupp et al. 1995). Because of this, the morphological apoptotic index in the samples was also evaluated, and it correlated significantly with the index determined by TUNEL method, suggesting reproducibility of the values obtained by the labeling. One might assume that enhanced apoptosis would retard tumor growth and be an important factor for a favorable prognosis compared to malignancies with low apoptotic activity. Previously, however, a high rate of apoptosis has been shown to associate with a shortened survival in non-small cell lung carcinoma (Törnänen et al. 1995), breast carcinoma (Vakkala et al. 1999), prostate carcinoma (Aihara et al. 1994) and transitional cell carcinoma of the urinary bladder (Lipponen & Aaltomaa 1994). The extent of apoptosis did not affect patient survival in HCC, gallbladder and pancreatic carcinomas. Apoptosis did not correlate with PCNA index in either HCC or pancreatic carcinoma.

6.3.1. The growth index

To evaluate the growth potential of HCC, a growth index from the scores obtained for apoptosis, necrosis and cell proliferation was designed. Patients whose tumors showed a high degree of proliferation relative to the degree of necrosis and apoptosis (i.e. had a positive growth index) had a significantly shorter survival and disease-free interval after operation than those with a tumor predominated by apoptosis and necrosis. When these factors were considered separately, no statistically significant association with survival time or post-operative disease-free interval was seen. The results show that assessment of growth potential by scoring all the relevant parameters may be of value in estimating patient prognosis. As these factors reflect the end results of the genetic and associated changes in tumors, their evaluation may be more practical than analysing the expression of different oncogenes or tumor suppressor genes, since several different cancer genes may be affected in a single tumor and all of them cannot usually be analysed at the same time. Probably the best clinically observable correlate of the growth index is growth rate,
a principal determinant of the aggressiveness of a tumor and an important prognostic factor. Tumors that had a positive growth index grew three times faster and relapsed three times more quickly than the others. This data supports the fact that the growth index is biologically relevant because it associates with the ultrasonographically determined tumor growth data obtained from the patients.

The value of a growth index was tested with HCC because these tumors contain only little stroma. A similar growth index was also designed for pancreatic adenocarcinoma in order to find out whether the results would be the same for this tumor group. Even though tumors with a high growth index had a bad outcome, the association was not significant. The main reason for this is that pancreatic carcinomas contain a lot of stromal tissue which makes it hard to compose a growth index in a reliable way. Yet another problem with the growth index is that cell mobility cannot be assessed. Surely tumors harboring mobile invasive cells might be larger also due to the migration of the neoplastic elements to the adjacent tissue.

### 6.4. The regulation of apoptosis

#### 6.4.1. p53

Because of the crucial role of p53 in the regulation of cell cycle, p53 overexpression was investigated in HCC, gallbladder and pancreatic carcinomas. Positive nuclear immunostaining for p53 was found in 23% of HCCs, which is about the same percentage as has been previously described in European populations (Volkmann et al. 1994). p53 positivity was here associated with a high proliferation index and a high growth index in HCC. This is as expected on the basis of the central role of p53 in the regulation of cell proliferation. Patients with p53-positive tumors had shorter survival than p53-negative ones, but the association was not statistically significant. Positive p53 immunostaining was seen in 57% of gallbladder carcinomas, which is in line with other studies (Sasatomi et al. 1996).

p53 overexpression was found in 41% of pancreatic carcinomas, which is in line with previous studies (Greenblatt et al. 1994). Cell proliferation measured by PCNA immunohistochemistry and mitotic count correlates with p53 overexpression. It has been shown that p53-induced cell cycle inhibition is accompanied by a selective down-regulation of PCNA mRNA and protein expression. The association between a high PCNA index and p53 overexpression may partly depend on this. The extent of necrosis was significantly associated with p53 overexpression in pancreatic carcinoma, which might relate to the high proliferation rate. This could lead to an increased necrosis because of the insufficient vascular supply in rapidly growing tumors. A similar association was seen in small cell lung carcinomas (Eerola et al. 1997). No association was found between p53 expression and apoptosis in either HCC, gallbladder or pancreatic carcinomas.
6.4.2. Expression of bcl-2 family proteins

The bcl-2 family is a group of apoptosis-regulating genes which can inhibit or promote apoptosis. Bcl-2, mcl-1 and bax expression was defined in this tumor material. Bcl-2 expression was found in only 13% of the pancreatic carcinomas. This figure is somewhat lower than the one reported by Sinicrope et al. (1996) who, in a series of 40 pancreatic tumors, found bcl-2 positivity in 55% of the cases. Frequent expression of bcl-2 has been found in epithelial tumors of other types, especially hormone sensitive carcinomas, such as breast and endometrial carcinomas, where expression is found in 70 to 80% of the cases (Mustonen et al. 1997, Saegusa et al. 1996). On the other hand, some other types of epithelial tumors, like non-small cell lung carcinoma, express bcl-2 only in a minority of cases (Törmänen et al. 1995). In gallbladder carcinomas bcl-2 expression was found in only 10% of the cases, suggesting that it does not play any significant role in the inhibition of apoptosis in this type of carcinoma. Only one HCC with positive immunohistochemistry for bcl-2 was found, which is in line with previous studies (Charlotte et al. 1994). The lack of bcl-2 immunoreactivity in the majority of pancreatic carcinomas could contribute to increased apoptosis because of an unopposed effect on the apoptosis-promoting bax. When bcl-2 was present, significantly lower apoptotic indices compared with other cases were found. The low expression of bcl-2 could be compensated with other apoptosis inhibiting proteins, such as survivin.

The anti-apoptotic mcl-1 was expressed in the majority of the pancreatic tumors and these cases had a slightly lower apoptotic index. According to the results, mcl-1 does not play a significant role in the regulation of apoptosis in pancreatic carcinoma. Most gallbladder tumors showed mcl-1 expression. Interestingly, tumors with a high mcl-1 expression seem to have a low bcl-2 expression, suggesting that mcl-1, instead of bcl-2, might play a more significant role in apoptosis inhibition in gallbladder tumors. No significant association was found between the extent of apoptosis and mcl-1 in gallbladder carcinomas and dysplasias, suggesting that other more powerful factors regulating apoptosis are also at play.

Bax expression was observed in all of the pancreatic tumors. In line with its apoptosis-promoting effect, tumors with a strong bax expression showed a significantly higher apoptotic index than tumors with a weaker index, suggesting that bax takes part in the promotion of apoptosis in them. Strong bax expression was found in most of the gallbladder carcinomas similar to many other types of epithelial tumors (Krajewski et al. 1994, Krajewska et al. 1996, Mustonen et al. 1997, Soini et al. 1998c). No significant association between apoptosis and the expression of bax was, however, seen.

6.4.3. Caspases 3, 6 and 8

Because the antibodies used recognized both the inactive pro-caspase and the activated cleaved caspases, the analysis had to be based only on the quantity of the immunostaining indicating the amount of protein in each specimen. Mainly cytoplasmic expression of caspases 3, 6 and 8 was seen in majority of pancreatic carcinomas. Membrane-associated reactivity of caspase 8 was also sometimes seen. This could be due to interaction with
membrane-associated death-signalling complexes, such as Fas-receptor complex, leading to a higher concentration of this caspase in the subplasmalemmal area (Muzio et al. 1997, Nagata 1997). Caspase 6, on the other hand, often displayed intensely stained granular cytoplasmic and sometimes also extracellular fragments reminiscent of apoptotic bodies, suggesting possible accumulation of caspases in such degrading apoptotic fragments. Similar staining was occasionally seen with caspase 8. Carcinoma cells for some reason increase the synthesis of caspases 3, 6 and 8 and are probably more susceptible to apoptosis than normal cells, which expressed only faint, occasional positivity for these caspases. The islands of Langerhans showed intense staining for caspase 8 as well as mcl-1, an anti-apoptotic member of the bcl-2 family. It might reflect the reaction of the cells of the neuroendocrine islands of Langerhans to an extrinsic apoptotic stimulus launched e.g. by surrounding inflammatory cells. No association was found between the apoptotic index and expression of caspases in pancreatic tumors.

In the present analysis of gallbladder carcinomas, strong expression of caspases 3, 6 and 8 was found in many tumors, and the immunoreactivity was clearly stronger than the expression observed in non-neoplastic or dysplastic gallbladder epithelium, suggesting that during neoplastic process, the synthesis and expression of caspases 3, 6 and 8 are upregulated. The increased expression of caspases in malignant cells compared to non-neoplastic ones may be one reason for the generally increased apoptotic activity in malignant tumors. No association between the expression of caspases 3, 6 and 8 and bcl-2 family proteins (bcl-2, mcl-1 and bax) was found. The expression of caspases 3, 6 and 8 was seen in a majority of HCCs. Caspase 3 staining was similar to the staining seen in pancreatic and gallbladder tumors as well as with caspases 6 and 8. No association was found between the expression of caspases and the extent of apoptosis.

6.5. Cell proliferation

Proliferation capacity is a principal determinant of the growth rate and aggressiveness of a tumor. The proliferating cell nuclear antigen (PCNA) and proliferation associated antigen Ki-67 have been used to determine cell proliferation in a variety of malignancies (Kawai et al. 1994, Törnänen et al. 1995, Pelosi et al. 1992). Synthesis of PCNA correlates directly with DNA replication and cell proliferation (Hall et al. 1990). Expression of PCNA increases in late G1, is maximal in S1 and then declines (Hall et al. 1990). Ki-67 is a DNA-binding nuclear protein which is another marker for cell proliferation. It is expressed throughout the cell cycle in proliferating but not in G0 cells (Gerdes et al. 1984). Analysis of Ki-67 has previously been restricted to frozen sections, due to the instability of the Ki-67 epitope in formalin fixation. MIB-1 is an antibody against Ki-67 and it is able to detect proliferating cells in paraffin-embedded tissue sections (Cattorelli et al. 1992).

In this study cell proliferation was measured by two parameters, PCNA immunohistochemistry and mitotic count. There was a positive correlation between the percentage of PCNA-positive cells and the frequency of mitoses in HCC. However, in pancreatic carcinomas no correlation was found. The reason for this may be that PCNA also takes part in DNA excision repair and is probably therefore variably activated in
malignant tumors (Hall et al. 1990, Shivji et al. 1992). In endocrine pancreatic tumors a high PCNA index has previously been shown to correlate with decreased mean survival (Pelosi et al. 1992). In our material of exocrine pancreatic adenocarcinomas, there was no association between a high PCNA index and patient prognosis, suggesting that the prognostic value of the PCNA index is different between endocrine and exocrine tumors of the pancreas.

6.6. P-glycoprotein in HCC

Pgp was mainly confined to the cell membrane in HCCs, a pattern also seen in the adjacent non-neoplastic liver cells. Intracellular globular immunostaining was observed in some cases, suggesting that Pgp was present in the Golgi apparatus, confirming previous electron microscopic studies (Dietel 1991). Increased expression of Pgp has been found in several epithelial tumors, such as those of the colon and breast (Cordon-Cardo et al. 1990, Verrelle et al. 1991, Sinicrope et al. 1994). Pgp was expressed in 65% of HCCs, which is in line with other studies (Chou et al. 1997). Previous studies have reported that Pgp positive tumors, such as those of the breast and colon, are associated with a shorter disease-free interval, as was the case in the present study with HCC (Verrelle et al. 1991, Sinicrope et al. 1994). In HCCs tumors showing Pgp positivity had a lower apoptotic index than tumors with no positivity, but this did not reach statistical significance.
7. Summary and conclusions

The growth of a tumor depends on the proliferative capacity and destruction of the tumor cells via apoptosis or necrosis. In the present study apoptosis and its regulatory proteins and their relation to other factors have been studied in highly malignant tumors of the liver, gallbladder and pancreas.

The results showed a relatively low apoptotic index in HCC, gallbladder and pancreatic carcinoma. An increase in the extent of apoptosis from dysplasias and grade I to grade II and III tumors was seen in gallbladder carcinomas. Of the bcl-2 family proteins, bcl-2 was infrequently expressed in all three tumor groups, while expression of mcl-1 and bax was frequent in gallbladder and pancreatic tumors. The anti-apoptotic bcl-2 and pro-apoptotic bax both regulate the extent of apoptosis in pancreatic carcinomas, while the role of the anti-apoptotic mcl-1 seems to be less important. In HCCs and gallbladder carcinomas the expression of the bcl-2 group proteins studied did not associate with apoptosis, suggesting that they do not play any significant pro- or antiapoptotic role in these tumors. Caspases 3, 6 and 8 were all strongly expressed in all three tumor cells studied and thus contribute to their susceptibility to undergo apoptosis. p53 overexpression was found to be quite at the same level in HCC, gallbladder and pancreatic carcinoma. Results on HCC showed that the capacity of a tumor to grow (growth index as determined by PCNA, apoptosis and necrosis scoring) was associated with a shortened survival in operated HCC. The results on growth index tended to be quite similar to HCC in pancreatic carcinoma. Evaluation of these parameters in tumors with a scant storma may be of value in assessing the prognosis of the disease. In HCCs P-glycoprotein associated with a shorter disease-free interval and shorter survival time. According to the results HCC, gallbladder and pancreatic carcinomas not only originate from organs sharing a common embryological derivation, but are also similar as far as apoptosis and its regulating proteins are concerned.
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