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Apoptosis in gallbladder carcinomas and dysplasias, its relation to the expression of caspases 3,6 and 8 and apoptosis regulating proteins bcl-2, mcl-l and bax

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Summary. In this study we investigated apoptosis and the expression of caspases 3, 6 and 8 and bcl-2, mcl-1 and bax in 39 gallbladder carcinomas and 7 epithelia1 dysplasias. The average apoptotic index was 0.68±0.91%. The extent of apoptosis was higher in grade II-III than grade I tumours or epithelial dysplasias (p=0.003). Also, tumours invading beyond serosa or into other organs (T3-T4) had a higher apoptotic index than other tumours (p=0.05). Caspase 3 expression was found in 37 (95%) and caspase 6 and 8 expression each in 30 (77%) carcinomas. Their expression associated with each other and tended to increase along with the progression of the lesions. Bcl-2 expression was found in only 4 (10%) tumours. In contrast, mcl-l positivity was found in 34 (87%) and bax positivity in all cases. The results show that apoptosis is increased along with progression of the neoplastic lesion of the gallbladder epithelium. Caspases 3,6 and 8 are strongly expressed in gallbladder carcinomas suggesting that they contribute to the increased apoptosis observed in them. Of the bcl-2 family proteins, bcl-2 was expressed infrequently suggesting that it does not play any significant role in apoptosis inhibition in gallbladder tumours.

Key words: Gallbladder, Apoptosis, Carcinoma, Caspase

Introduction

Apoptosis is a biochemically regulated cell death program defined by characteristic morphological and biochemical changes (Kerr et al., 1994; White, 1996; Yang and Korsmeyer, 1996; Kroemer, 1997). Unlike necrosis it usually involves scattered cells with no associated inflammation (Kerr et al., 1994). The onset of apoptosis is characterised by nuclear shrinkage, compaction and segregation of chromatin against the nuclear envelope and condensation of the cytoplasm followed by nuclear fragmentation into several membrane-bound apoptotic bodies (Kerr et al., 1994). Apoptotic cells or bodies are then phagocytized by adjacent macrophages or parenchyma1 cells (Kerr et al., 1994).

Biochemically, apoptosis is characterised by fragmentation of DNA into periodical 180 bp fragments which can be detected in DNA electrophoresis as a typical ladder pattern (Kerr et al., 1994). The DNA fragmentation and nuclear destruction is caused by caspases (Alnemri et al., 1996; Soini et al., 1998a; Thornberry and Lazebmik, 1998) These are proteolytic enzymes which are able to cleave proteins with a consensus sequence at aspartatic acid-alanine residues (Thornberry et al., 1994; Alnemri et al, 1996; Patel et al., 1996; Barge et al., 1997; Harvey et al., 1997; Thornberry and Lazebnik, 1998). Currently, at least 13 different caspases are known in mammalian cells (Thornberry and Lazebnik, 1998). They reside in the cytosol as inactive forms and need to be activated in order to perform their function (Thornberry et al., 1994; Harvey et al., 1997; Thornberry and Lazebnik, 1998). Cleavage of the aminoterminal portion followed by fragmentation of the rest of the molecule into 10 and 20 kDa fragments leads to their autocatalytic activation (Thornberry et al., 1994; Thornberry and Lazebnik, 1998). In addition to activating themselves, activated caspases are also able to cleave several other proteins (Patel et al., 1996). These include enzymes of the DNA repair system, such as poly(ADP-ribose)-polymerase, many structural proteins such as nuclear lamins, fodrin, ß-katenin, cytokeratin 18 and some oncoproteins such as retinoblastoma protein and mdm2 (Patel et al., 1996; Rao et al., 1996; Brancolini et al., 1997; Caulin et al., 1997; Chen et al., 1997; Tan et al., 1997). Caspases may be activated directly from the cell membrane through ligand binding of TNF receptors such as APO1/FAS (Muzio et al., 1996; Nagata, 1997). Binding of APO1/FAS receptor to its FAS ligand leads to oligomerization of the receptor and to formation of the death-inducing signalling complex including the receptor, FADD/MORT1 and procaspase 8 (FLICE) (Muzio et al., 1996; Nagata, 1997).

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Activation of caspases can also be brought about indirectly through changes in the membrane potential and pore formation of mitochondria which leads to release of caspase activating substances such as cytochrome c or apoptosis inducing factor (AIF) from them (Kluck et al., 1997; Manon et al., 1997; Yang et al., 1997). Important in the modulation of the apoptotic response are proteins of the bcl-2 family which have been shown to be able to influence the mitochondrial membrane potential or are able to form pores in them (Kluck et al., 1997; Manon et al., 1997; Minn et al., 1997; Yang et al., 1997). The bcl-2 group of proteins may either be pro- or antiapoptotic (White, 1996; Yang and Korsmeyer, 1996; Kroemer, 1997; Adams and Cory, 1998). Proapoptotic members include bax, bcl-xS and bad and anti-apoptotic bcl-2, bcl-xL and mcl-1 (White, 1996; Yang and Korsmeyer, 1996; Kroemer, 1997; Adams and Cory, 1998). Bcl-2 and bcl-xL have been shown to inhibit release of cytochrome c or AIF from mitochondria while bax promotes it (Kluck et al., 1997; Manon et al., 1997; Yang et al., 1997; Minn et al., 1997). Even though the bcl-2 group of proteins may in this way modulate caspase activity, also caspases may modulate the proteins of the bcl-2 group and in this way may influence apoptosis. Caspase 3 has been shown to be able to cleave bcl-2 and the truncated fragment has been shown to be proapoptotic (Fujita and Tsuruo, 1998). Similarly, the anti-apoptotic bcl-xL is modulated through interaction with caspases resulting in its cleavage to a similar truncated pro-apoptotic fragment (Clem et al., 1998).

Of the apoptosis-regulating proteins, bcl-2 has been most extensively studied in clinical tumor material. Due to a 14;18 translocation bcl-2 is frequently over-expressed in follicular lymphomas and may thus act as an oncogene in those tumours (Tsujimoto et al., 1985; Kiberu et al., 1996; Soini et al., 1998b). In epithelial tumours, the expression of bcl-2 is varied. Some tumours, such as breast or endometrial carcinomas display a high expression while other epithelial tumours, such as carcinomas of the liver or non-small cell lung carcinomas, show a lower expression (Törmänen et al., 1995; Saegusa et al., 1996; Mustonen et al., 1997; Soini et al., 1998a). Other members of the bcl-2 group of proteins are less extensively studied. Bax, however, seems to show a strong expression in tumours and it seems to be more or less constitutively expressed (Krajewski et al., 1994; Krajewska et al., 1996a,b; Soini et al., 1998a-c). Mcl-1 is many times strongly expressed in tumours which show a low bcl-2 expression (Krajewska et al., 1996b). This can also be seen in non-neoplastic tissues, where mcl-1 was expressed in chondroid and striated muscle which lack bcl-2 expression (Krajewski et al., 1995). There are no previous immunohistochemical studies on caspase expression in epithelial tumours. In malignant lymphomas, however, caspase 3 was found to be strongly expressed in large cell non-Hodgkin's lymphomas (Krajewski et al., 1997).

In order to investigate programmed cell death in neoplastic gallbladder epithelium we analysed the extent of apoptosis and the expression of caspases 3, 6 and 8 and bcl-2, mcl-1 and bax in 53 cases of gallbladder lesions consisting of 39 adenocarcinomas, 7 epithelial dysplasias and 7 non-neoplastic lesions. The results were correlated with the clinical data of the patients. Additionally, the extent of necrosis was determined from the cases and the reproducibility of the results on apoptosis was confirmed by also calculating the apoptotic index by light microscopic morphology.

Materials and methods

Materials

39 surgically resected gallbadder carcinomas, 7 dysplasias (one mild, three moderate and three severe) and 7 cases of non-neoplastic gallbladder samples were collected from the files of the Department of Pathology, Oulu University Hospital between 1986 and 1995. All tissue material had been embedded in paraffin and fixed in 10% neutral formalin. The diagnosis of all cases was based on conventional light microscopy according to the criteria of the World Health Organization (Albores-Saavedra et al., 1991). 13 of the tumours were grade III, 13 of grade II and 13 of grade I. The case histories of all the patients were reviewed and the pertinent clinical data, including the survival, age and sex of the patients were collected from the hospital records. 14 patients received chemotherapy, usually mitomycin. The average age of the patients was 69.2±12.3 years.

3'-end labelling of DNA in apoptotic cells

In order to detect apoptotic cells, *in situ* labelling of the 3'-ends of the DNA fragments generated by apoptosis-associated endonucleases was performed using the ApopTag *in situ* apoptosis detection kit (Oncor, Gaithersburg, MD, USA) as previously described (Törmänen et al., 1995; Soini et al., 1996). The sections, after being dewaxed in xylene and rehydrated in ethanol, 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 PBS, pH 7.2. The slides were then treated with terminal transferase enzyme and digoxigenin-labelled nucleotides after which anti-digoxigenin-peroxidase solution was applied on the slides. The colour was developed with diaminobenzidine after which the slides were lightly counterstained with hematoxylin. For control purposes we used tissue sections from hyperplastic lymph nodes showing an increased number of apoptotic B cells within germinal centers and a low number of apoptotic T cells in the interfollicular areas.

Assessment of the apoptotic index

Cells were defined as apoptotic if the whole nuclear

area of the cell labelled positively. Apoptotic bodies were defined as small positively-labelled globular bodies in the cytoplasm of the tumour cells which could be found either singly or in groups. To estimate the apoptotic index (the percentage of apoptotic events in a given area), apoptotic cells and bodies were counted in 10 high-power fields (HPFs) and this figure was divided by the number of tumour cells in the same HPFs.

Morphological assessment of apoptosis

The morphological assessment of apoptosis was made in routinely stained haematoxylin-eosin sections following the same principles as outlined above. The morphological criteria for apoptosis have been previously described (Kerr et al., 1994). Apoptosis was not assessed in the vicinity of necrotic areas with both the 3'-end labelling or morphology.

Assessment of necrosis

The extent of necrosis was assessed light microscopically by evaluating the proportion of necrotic areas in tumor tissue. This was performed by assessing the proportion of necrotic areas in tumor tissue using a x40 magnification and going through all areas in all available tissue sections. The percentage of necrosis was the sum proportion of necrosis/all tumor tissue.

Immunohistochemical stainings

A monoclonal antibody (clone 124) against bcl-2 oncoprotein was obtained from Dako (Glostrup, Denmark). Polyclonal antibodies to mcl-1 and bax were obtained from Pharmingen (San Diego, California, USA). Polyclonal rabbit anti-human caspase-3 antibody was purchased from Pharmingen (San Diego, California, USA). According to the manufacturer, the antibody recognizes both the unprocessed 32 kDa pro-caspase-3 molecule and the fragmented larger active 17 kDa unit. Polyclonal goat anti-human antibodies to caspase 6 (mch2) and caspase 8 (mch5) were purchased from Santa Cruz Biotechnology, Inc (Santa Cruz, California, USA). According to the manufacturer, the mch2 antibody recognizes amino acids 157-176 and mch5 amino acids 354-373 of the carboxy terminal part of the proteins which both belong to the processed p20 fragment of the protein.

Before application of the primary antibodies, the sections were heated in a microwave oven in 10 mM citric acid monohydrate, pH 6.0, for 10 minutes. After incubation with the primary antibody (dilution 1:50 for 30 min for bcl-2, 1:1000 overnight for bax, 1:750 overnight for mcl-1, 1:500 for 60 min for anti-caspase 3, 1:100 for 60 min for anti-caspases 6 and 8), a biotinylated secondary anti-mouse, anti-rabbit or anti-goat antibody (all three from Dakopatts, Copenhagen, Denmark) was applied (dilution 1:200) followed by the avidin-biotin-peroxidase complex (Dakopatts).

For all the immunostainings, the colour was developed by diaminobenzidine, whereafter the sections were lightly counterstained with methyl 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. As a positive control for the immunostainings, a lymph node with follicular hyperplasia was used.

The intensity of the immunostainings with all the antibodies was evaluated by dividing the staining reaction into four groups: 1) weak cytoplasmic staining intensity; 2) moderate cytoplasmic staining intensity; 3) strong cytoplasmic staining intensity; or 4) very strong cytoplasmic staining intensity.

The quantity of the immunostaining was evaluated as follows; 0 (no positive immunostaining), 1 (<25% of tumor cells showing cytoplasmic positivity), 2 (25-50% of tumor cells showing cytoplasmic positivity), 3 (50-75% of tumor cells showing cytoplasmic positivity), or 4 (>75% of tumor cells showing cytoplasmic positivity),

A combined score for the immunostaining, based on both qualitative and quantitative immunostaining was composed by adding both the qualitative and quantitative score which was then divided into three main groups; 0) no immunostaining; score 0, 1) weak immunostaining; score 1-4; and 2) strong immunostaining; score 5-8.

Statistical analysis

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 correlation analysis. The survival data was analysed according to the Kaplan-Meyer method. The difference between survival in different groups was analysed using the log rank, Breslow and Tarone-Ware tests. Probability values of less than 0.05 were considered significant.

Results

Apoptosis in gallbladder carcinomas

The average apoptotic index in gallbladder carcinomas and dysplasias was 0.68±0.91% as determined by the TUNEL method and 0.59±0.67% according to morphological evaluation (Fig. 1). There was a statistically significant correlation between the apoptotic index as determined by the TUNEL method and morphological evaluation by light microscopy (R=0.3456, p=0.022). The apoptotic indices in grade I, II and III carcinomas and in dysplasias are shown separately in Table 1. There was a significantly lower extent of apoptosis in dysplasias and grade I tumours than in grade II-III tumours (p=0.003). The apoptotic index also correlated with necrosis (R=0.4378, p=0.002) and grade III tumours displayed more necrosis than other

tumours (p=0.05). The extent of apoptosis was lower in dysplasias and T1-T2 tumours than in T3-T4 tumours (p=0.05). No significant association was found between patient survival and apoptosis in carcinomas even though patients with tumours of a higher extent of apoptosis tended to have a worse outcome (p=0.16, log rank).

Immunohistochemistry of caspases 3, 6 and 8

Weak occasional positive caspase 3,6 and 8 intracytoplasmic positivity was seen in the non-neoplastic gallbladder epithelium. In areas with inflammation, the epithelium seemed to express caspases more strongly. In neoplastic epithelium the caspase immunoreactivity progressively increased according to the severity of the lesion and was strongest in grade II-III lesions (see Table 2). The immunoreactivity for all caspases was mostly diffuse cytoplasmic, but with caspases 6 and 8 also granular cytoplasmic positivity was seen (Figs. 2, 3). Interestingly, in one case of a gallbladder carcinoma also nuclear caspase 3 positivity was observed (Fig. 2). There was no statistically significant association between no and weak or strong caspase 3, 6 and 8 immunoreactivity and apoptosis (p=0.24, p=0.98 and p=0.82, respectively). Similarly, there was no statistically significant association between no and weak or strong caspase 3, 6 and 8 immunoreactivity and necrosis (p=0.51, p=0.44 and p=0.94, respectively). On the other hand, there was a strong statistical association between the expression of the 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). Strong caspase 3, 6 or 8 expression in tumours did not associate with the survival of the patients in gallbladder carcinoma (p=0.78, p=0.16 and p=0.17, respectively). Neither was there any association between chemotherapy treatment and strong caspase 3, 6 or 8 immunoreactivity in the tumours (p=0.70, p=0.17 and p=0.08, respectively).

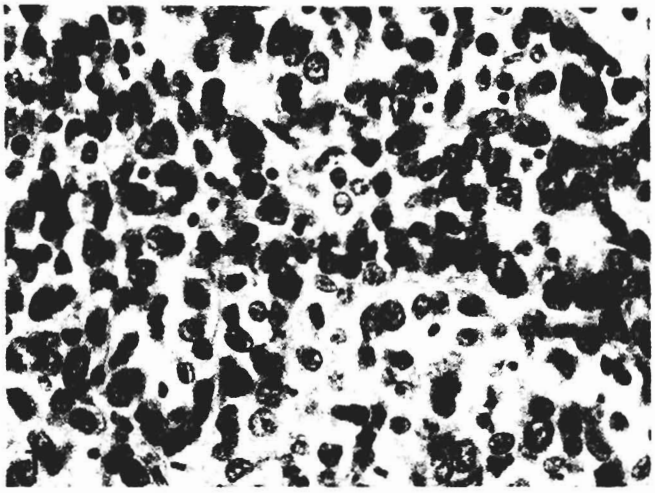

Fig. 1. Apoptosis in gallbladder carcinoma. In this grade III adenocarcinoma, two positively-stained apoptotic cells can be seen among tumor cells in the middle field. x 400

Table 1. Extent of apoptosis as determined by the TUNEL method and by morphology and percentage of necrosis in gallbladder dysplasias and carcinomas

LESION	APOPTOSIS & BY TUNEL	MORPHOLOGIC APOPTOSIS	NECROSIS %
Normal	0.02±0.02	0.02±0.03	0.00±0.00
Dysplasia	0.06±0.10	0.14±0.11	0.00±0.00
Grade I carcinoma	0.53±0.80	0.42±0.41	6.62±1.84
Grade II carcinoma	0.64±0.80	0.67±0.43	2.14±2.63
Grade III carcinoma	1.34±1.11	0.98±0.98	13.40±18.47

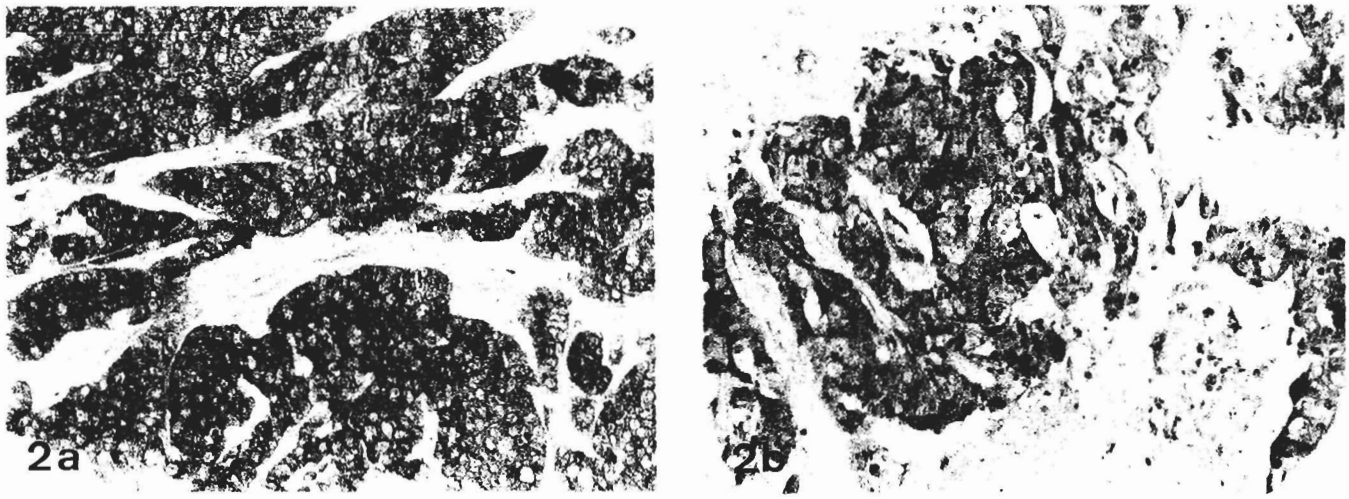

Fig. 2. Caspase 3 immunoreactivity in gallbladder carcinoma. **a.** The tumor cells show diffuse cytoplasmic immunoreactivity for caspase 3. **b.** In some areas nuclear positivity could also be seen. x 200

Immunohistochemistry of bcl-2, mcl-1 and bax

In non-neoplastic gallbladder epithelium no bcl-2 positivity was observed. In contrast, relatively strong mcl-1 or bax expression was seen in the non-neoplastic epithelium, especially in areas with inflammation. In the neoplastic lesions, bcl-2 expression was detected in 4/39 (10%) carcinomas and in 1/7 (14%) dysplasias. Most tumours and dysplasias showed mcl-1 and all bax expression. In only five tumours, no evident mcl-1 expression was seen. Strong bax immunoreactivity was seen in 22/39 (56%) of the tumours while strong mcl-1 expression was seen in only 5/39 (13%) of the cases. In dysplasias, 3 cases showed strong mcl-1 or bax expression (43%). There was no association between the extent of apoptosis and the expression of bcl-2, mcl-1 or bax in gallbladder carcinomas (p=0.15, p=0.45, p=0.53, respectively). Neither was there any association between tumour grade, necrosis, survival, the expression of caspases and the expression of the bcl-2 group of proteins (data not shown).

Discussion

This study was undertaken to analyse apoptosis in gallbladder carcinomas and dysplasias and to compare the extent of apoptosis with the immunohistochemical expression of caspases 3, 6 and 8 and the apoptosis regulating proteins bcl-2, bax and mcl-1. The data was correlated with the grade and the stage of the tumours and the survival of the patients. The average extent of apoptosis in gallbladder carcinomas was 0.84% which is within the same range as has been observed in several other types of carcinomas (for a review, see Soini et al., 1998a).

According to our results there is a progressive increase in the extent of apoptosis from dysplasias to high grade tumours of the gallbladder (see Table 1). The results are in line with observations from other epithelial tumours, 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 tumour progression. Such apoptosis-regulating genes include p53, Rb, ras and c-myc, the expression of which may be changed during tumour progression (Soini et al., 1998a). Also the anti-apoptotic bcl-2 may act as an oncogene. In follicular lymphomas, a 14;18 translocation leads to activation of the bcl-2 gene and overexpression of the protein product (Tsujimoto et al., 1985). In epithelial tumours bcl-2 has

Table 2. Expression of caspases 3, 6 and 8 and bcl-2, bax and mcl-1 in gallbladder epithelial dysplasias and carcinomas.

LESION	IMMUNOHISTOCHEMICAL EXPRESSION (no expression/weak expression/strong expression)					
	caspase 3	caspase 6	caspase 8	bcl-2	bax	mcl-1
Normal epithelium	5/1/1	7/0/0	6/1/0	7/0/0	0/3/4	3/2/3
Dysplasias	0/6/1	3/3/1	3/3/1	7/0/0	0/4/3	0/4/3
Grade I carcinoma	0/9/4	4/8/1	5/7/1	13/0/0	0/6/7	4/6/3
Grade II carcinoma	1/8/4	2/8/3	1/8/4	13/0/0	0/4/9	0/10/3
GradeIII carcinoma	1/5/7	3/7/3	3/6/4	9/4/0	0/7/6	1/12/0

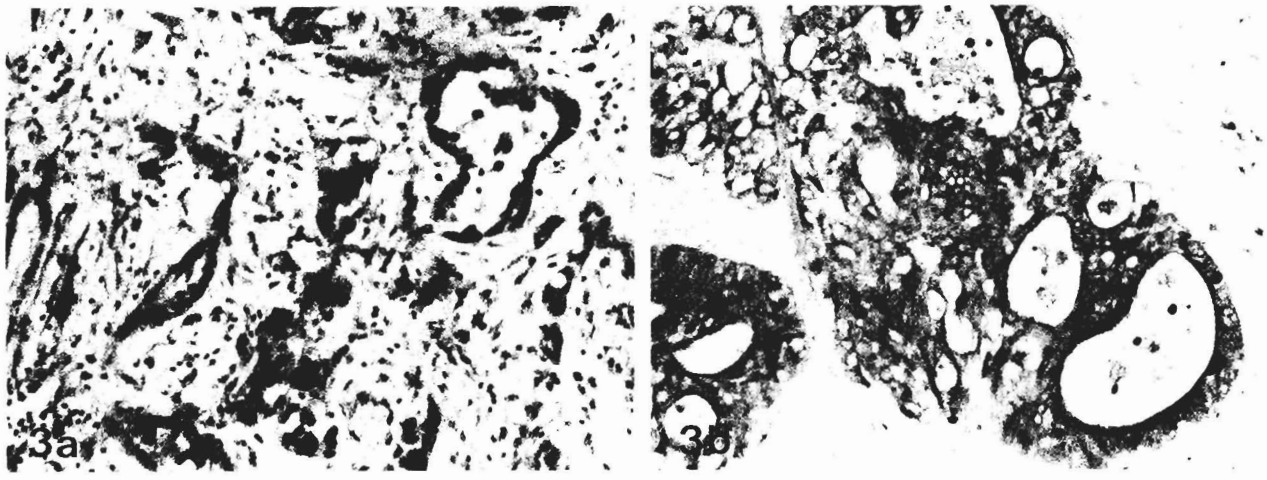

Fig. 3. Caspase 6 and 8 immunoreactivity in gallbladder carcinomas. Strong cytoplasmic positivity can be seen for caspase 6 **(a)** and 8 **(b)** in the tumor cells. x 200

not, however, been shown to be translocated even though the protein is frequently overexpressed in many tumours, such as breast or endometrial carcinomas (Saegusa et al., 1996; Mustonen et al., 1997; Soini et al., 1998a).

In addition to tumour 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 may partly be commonly regulated. It has been shown that stimuli causing apoptosis at low doses may cause necrosis when the doses are high (Leist and Nicotera, 1997). Also, many stimuli causing apoptosis (e.g. heat shock, hypoxia, viruses, radiation, nitric oxide, etc.) also may cause necrosis (Leist and Nicotera, 1997). At the biochemical level, depletion of intracellular ATP in human T cells shifts cell death from apoptosis to necrosis (Leist et al., 1997). It has also been shown that the reaction of the cell to a destructive stimulus depends on the concentration of endogenous caspases in the cytosol (Green and Reed, 1998). If the concentration of caspases is low, the cell reacts by necrosis, if it is high, the caspase cascade is preferentially activated leading to apoptosis (Green and Reed, 1998). The statistical association between apoptosis and necrosis in gallbladder carcinoma might thus reflect the close mechanistic association between these phenomena. On the other hand, the association may also be due to methodological aspects; necrotic cells have also been reported to be labelled by the TUNEL method (Grasl-Kraupp et al., 1995). Because of this, we also evaluated the morphological apoptotic index in the samples. It significantly correlated with the apoptotic index determined by TUNEL suggesting reproducibility of the values obtained by TUNEL.

In gallbladder carcinomas bcl-2 expression was found in only 10% suggesting that its expression does not play any significant role in the inhibition of apoptosis in gallbladder carcinomas. The expression is similar to some other tumours, such as prostate or hepatocellular carcinomas, which also show a low bcl-2 expression (Krajewska et al., 1996b; Soini et al., 1998a). The low bcl-2 expression in gallbladder tumours might, however, implicate some derangement in the apoptotic regulating apparatus and might thus contribute to an increased apoptosis found in epithelial tumours compared to the non-neoplastic cells. In line with this, bcl-2 expression has been shown to be inversely associated with apoptosis in many tumour types, such as non-Hodgkin's lymphomas, breast and salivary gland carcinomas (Kiberu et al., 1996; Mustonen et al., 1997; Soini et al., 1998b,c).

Contrary to bcl-2, expression of mcl-1, another anti-apoptotic protein, was found in most of the tumours. Interestingly, tumours 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 these tumours. In prostate cancer, for instance, bcl-2 expression was found in only 25%, while mcl-1 was expressed in 81% of cases (Krajewska et al., 1996b). We did not, however, find any significant association 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. Neither did we find any significant association between the expression of bax and apoptosis. Strong bax expression was, however, observed in most of the tumours suggesting that it is more or less constitutively expressed in gallbladder carcinomas. A similar strong bax expression is also observed in many other types of epithelial tumours (Krajewski et al., 1994; Krajewska et al., 1996a; Mustonen et al., 1997; Soini et al., 1998b,c).

While the bcl-2 group of proteins modulate the apoptotic response, caspases act as executioners of apoptosis. They are synthesised by several types of cells but their immunohistochemical expression in different types of epithelial tumours has not been extensively studied. In our analysis of gallbladder carcinomas, strong expression of caspases 3, 6 and 8 was found in many tumours and the immunoreactivity was clearly stronger than the expression observed in non-neoplastic or dysplastic gallbladder epithelium. This suggests that during the neoplastic process, the synthesis and expression of caspases 3, 6 and 8 are upregulated. In previous studies caspase 3 immunoreactivity has been detected in follicular non-Hodgkin's lymphomas where large cell high grade tumours expressed strongest caspase 3 activity (Krajewski et al., 1997). The increased expression of caspases in malignant cells compared to non-neoplastic epithelia may thus be one reason for the generally increased apoptotic activity in malignant tumours. In fact, a high endogenous caspase expression might partly protect the neoplastic cells from necrotic destruction by shifting the reaction of the cell induced by noxious environmental stimuli to apoptosis and in this way indirectly contribute to a smaller damage on the tumour tissue because apoptotic cell death only involves scattered cells and is not associated with inflammation and leakage of proteolytic enzymes from the damaged cells as happens in necrosis. This could also partly explain the association of a high extent of apoptosis with tumours of a higher grade as was also observed in our cases and also some observations where a high extent of apoptosis in tumours is paradoxically associated with a worse prognosis of the patients (Törmänen et al., 1995; Soini et al., 1998a).

There was no association between the expression of caspases 3, 6 and 8 and the three proteins of the bcl-2 family in gallbladder carcinoma. This suggests that the modulative effect of bcl-2, mcl-1 and bax on apoptosis in gallbladder carcinoma does not directly influence the synthesis or expression of these proteins. On the other hand, some caspases are known to be able to cleave antiapoptotic proteins of the bcl-2 family and the shorter truncated fragments have proapoptotic activity (Clem et al., 1998; Fujita and Tsuruo, 1998). An increased caspase activity might also in this way contribute to the increased apoptotic activity observed in many malignant

tumours. Little is known about the regulation of caspase synthesis. The association of the expression of caspases 3, 6 and 8, however, suggests that their synthesis is mutually regulated. In previous studies, ischemia has been shown to induce caspase 2 mRNA expression and some chemotherapeutic agents, such as etoposide, upregulate the synthesis of some other caspases (Harvey et al., 1997; Droin et al., 1998). In our study chemotherapy treatment did not, however, significantly associate with the expression of caspases in gallbladder carcinomas.

In conclusion, our results show that apoptosis is progressively increased in neoplastic gallbladder epithelium along with the progression of the neoplastic process. The expression of caspase 3, 6 and 8 is similarly increased. Of the bcl-2 family proteins, bcl-2 is infrequently expressed in gallbladder carcinomas while expression of mcl-1 and bax is frequent. No significant association could be observed between apoptosis and the expression of the proteins of the bcl-2 family or caspases suggesting a complex and multifaceted regulation for apoptosis in neoplastic gallbladder epithelial cells.

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References

Adams J.M. and Cory S. (1998). The bcl-2 protein family: arbitrers of cell survival. Science 281, 1322-1326.

Albores-Saavedra J., Henson D.E. and Sobin L.H. (1991). Histological typing of tumours of the gallbladder and extrahepatic bile ducts. World Health Organization. Second Edition. International Histological Classification of Tumours. Springer-Verlag, Berlin.

Alnemri E.S., Livingston D.J., Nicholson D.W., Salvesen G., Thornberry N.A., Wong W.W. and Yuan J. (1996). Human ICE/CED-3 protease nomenclature. Cell 87, 171.

Barge R.M.Y., Willemze R., Vandenabeele P., Fiers W. and Beyaert R. (1997). Differential involvement of caspases in apoptosis of myeloid leukemic cells induced by chemotherapy versus growth factor withdrawal. FEBS Lett. 409, 207-210.

Brancolini C., Lazarevic D., Rodriguez J. and Schneider C. (1997). Dismantling cell-cell contacts during apoptosis is coupled to a caspase-dependent proteolytic cleavage of ß-catenin. J. Cell. Biol. 139, 759-771.

Caulín C., Salvesen G.S. and Oshima R.G. (1997). Caspase cleavage of keratin 18 and reorganization of intermediate filaments during epithelial cell apoptosis. J. Cell Biol. 138, 1379-1394.

Chen L., Marechal V., Moreau J., Levine A.J. and Chen J. (1997). Proteolytic cleavage of the mdm2 oncoprotein during apoptosis. J. Biol. Chem. 272, 22966-22973.

Clem R.J., Cheng E.H., Karp C.L., Kirch D.G., Ueno K., Takahashi A., Kastan M.B., Griffin D.E., Earnshaw W.C., Veliuona M.A and Hardwick J.M. (1998). Modulation of cell death by Bcl-xL through caspase interaction. Proc. Natl. Acad. Sci. USA 95, 554-559.

Droin N., Dubrez L., Eymin B., Renvoize C., Breard J., Dimanche-Boitrel M.T. and Solary E. (1998). Upregulation of CASP genes in human tumor cells undergoing etoposide-induced apoptosis. Oncogene 16, 2885-2894.

Fujita N. and Tsuruo T. (1998). Involvement of bcl-2 cleavage in the acceleration of VP-16-induced U937 cell apoptosis. Biochem. Biophys. Res. Commun. 246, 484-488.

Grasl-Kraupp B., Ruttkay-Nedecky B., Koudelka H., Bukowska K., Bursch W. and Schulte-Hermann R. (1995). In situ detection of fragmented DNA (TUNEL assay) fails to discriminate among apoptosis, necrosis, and autolytic cell death: A cautionary note. Hepatology 21,1465-1468.

Green D.R. and Reed J.C. (1998). Mitochondria and apoptosis. Science 281, 1309-1312.

Harvey N.L., Butt A.J. and Kumar S. (1997). Functional activation of Nedd2/ICH-1 (Caspase-2) is an early process in apoptosis. J. Biol. Chem. 272, 13134-13139.

Kerr J.F.R., Winterford C.M. and Harmon B.V. (1994). Apoptosis. Its significance in cancer and cancer therapy. Cancer 73, 2013-2026.

Kiberu S.W., Pringle J.H., Sobolewski S., Murphy P. and Lauder I. (1996). Correlation between apoptosis, proliferation and bcl-2 expression in malignant non-Hodgkin's lymphoma. J. Clin. Pathol. Mol. Pathol. 49, M268-M272.

Kluck R.M., Bossy-Wetzel E., Green D.R. and Nwemeyer D.D. (1997). The release of cytochrome c from mitochondria: a primary site for bcl-2 regulation of apoptosis. Science 275, 1132-1136.

Krajewska M., Fenoglio-Preiser C.M., Krajewski S., Song K., MacDonald J.S., Stemmerman J.S. and Reed J.C. (1996a). Immunohistochemical analysis of bcl-2 family proteins in adenocarcinomas of the stomach. Am. J. Pathol. 149, 1449-1457.

Krajewska M., Krajewski S., Epstein J.I., Shabaik A., Sauvageot J., Song K., Kitada S. and Reed J.C. (1996b). Immunohistochemical analysis of bcl-2, bax, bcl-X and mcl-1 expression in prostate cancer. Am. J. Pathol. 148, 1567-1576.

Krajewski S., Krajewska M., Shabaik A., Miyashita T., Wang H.G. and Reed J.C. (1994). Immunohistochemical determination of in vivo distribution of bax, a dominant inhibitor of bcl-2. Am. J. Pathol. 145, 1323-133.

Krajewski S., Bodrug S., Krajewska M., Shabaik A., Gascoyne R., Berean K. and Reed J.C. (1995). Immunohistochemical analysis of mcl-1 protein in human tissues. Differential regulation of mcl-1 and bcl-2 protein production suggests a unique role for mcl-1 in control of programmed cell death in vivo. Am. J. Pathol. 146, 1309-1319.

Krajewski S., Gascoyne R.D., Zapata J.M., Krajewska M., Kitada S., Chhanabhai M., Horsman D., Berean K., Piro L.D., Fugier Vivier I., Liu Y.J., Wang H.G. and Reed J.C. (1997). Immunolocalization of the ICE/Ced-3-family protease, CPP32 (Caspase-3) in non-Hodgkin's lymphomas, chronic lymphocytic leukemias, and reactive lymph nodes. Blood 89, 3817-3825.

Kroemer G. (1997). The proto-oncogene bcl-2 and its role in regulating apoptosis. Nature Med. 3, 614-620.

Leist M. and Nicotera P. (1997). The shape of cell death. (Breakthroughs and views). Biochem. Biophys. Res. Commun. 236, 1-9.

Leist M., Single B., Castoldi A.F., Kühnle S. and Nicotera P. (1997). Intracellular adenosine triphosphate (ATP) concentration: A switch in the decision between apoptosis and necrosis. J. Exp. Med. 185, 1481-1486.

Manon S., Chaudhuri B. and Guerin M. (1997). Release of cytochrome c and decrease of cytochrome c oxidase in bax-expressing yeast cells, and prevention of these effects by coexpression of bcl-xL. FEBS Lett. 415, 29-32.

Minn A.J., Vélez P., Schendel S.L., Liang H., Muchmore S.W., Fesik S.W., Fill M. and Thompson C.B. (1997). Bcl-xL forms an ion channel in synthetic lipid membranes. Nature 385, 353-357.

Mustonen M., Raunio H., Pääkkö P. and Soini Y. (1997). The extent of apoptosis is inversely associated with bcl-2 expression in premalignant and malignant breast lesions. Histopathology 31, 347-354.

Muzio M., Chinnaiyan A.M., Kischkel F.C., O'Rourke K., Shevchenko A., Ni J., Scaffidi C., Bretz J.D., Zhang M., Gentz R., Mann M., Krammer P.H., Peter M.E. and Dixit V.M. (1996). FLICE, a novel FADD-Homologous ICE/CED-3-like protease, is recruited to the CD95 (Fas/APO-1) death-inducing signaling complex. Cell 85, 817-827.

Nagata S. (1997). Apoptosis by death factor. Cell 88, 355-365.

Patel T., Gores G.J. and Kaufmann S.H. (1996). The role of proteases during apoptosis. FASEB J. 10, 587-597.

Rao L., Perez D. and White E. (1996). Lamin proteolysis facilitates nuclear events during apoptosis. J. Cell Biol. 135, 1441-1455.

Saegusa M., Kamata Y., Isono M. and Okayasu I. (1996). Bcl-2 expression is correlated with a low apoptotic index and associated with progesterone receptor immunoreactivity in endometrial carcinomas. J. Pathol. 180, 275-282.

Soini Y., Virkajärvi N., Lehto V-P. and Pääkkö P. (1996). Hepatocellular carcinomas with a high proliferation index and a low degree of apoptosis and necrosis are associated with a shortened survival. Br. J. Cancer 73, 1025-1030.

Soini Y., Pääkkö P. and Lehto V-P. (1998a). Histopathological evaluation of apoptosis in cancer. A Review. Am. J. Pathol. 153, 1041-1053.

Soini Y., Raunio H. and Pääkkö P. (1998b). High grade malignant non-Hodgkin's lymphomas differ from low grade lymphomas in the extent of apoptosis and their expression of bcl-2, mcl-1, bax and p53. Tumor Biol. 19, 176-185.

Soini Y., Törmänen U. and Pääkkö P. (1998c). Apoptosis is inversely related to bcl-2 but not to bax expression in salivary gland tumours. Histopathology 32, 28-34.

Tan X., Martin S.J., Green D.R. and Wang J.Y.J. (1997). Degradation of retinoblastoma protein in tumor necrosis factor-α and CD95-induced cell death. J. Biol. Chem. 272, 9613-9616.

Thornberry N.A. and Lazebnik Y. (1998). Caspases: enemies within. Science 281, 1312-1316.

Thornberry N.A., Peterson E.P., Zhao J.J., Howard A.D., Griffin P.R. and Chapman K.T. (1994). Inactivation of interleukin-1ß converting enzyme by peptide (acyloxy)methyl ketones. Biochemistry 33, 3934-3940.

Tsujimoto Y., Cossman J., Jaffe E. and Croce C. (1985). Involvement of the bcl-2 gene in human follicular lymphoma. Science 228, 1440-1443.

Törmänen U., Eerola A-K., Rainio P., Vähäkangas K., Soini Y., Sormunen R., Bloigu R., Lehto V-P. and Pääkkö P. (1995). Enhanced apoptosis predicts shortened survival in non-small cell lung carcinoma. Cancer Res. 55, 5595-5602.

Törmänen U., Nuorva K., Soini Y. and Pääkkö P. (1999). Apoptotic activity is increased in parallel with the metaplasia-dysplasia-carcinoma sequence of the bronchial epithelium. Br. J. Cancer 79, 996-1002.

White E. (1996). Life, death, and the pursuit of apoptosis. A review. Gene Dev. 19, 1-15.

Yang E. and Korsmeyer S.K. (1996). Molecular thanatopsis: A discourse on the bcl-2 family and cell death. Blood 88, 386-401.

Yang J., Liu X., Bhalla K., Kim CN., Ibrado A.M., Cai J., Peng T-I., Jones D.P. and Wang X. (1997). Prevention of apoptosis by bcl-2: Release of cytochrome c from mitochondria blocked. Science 275, 1129-1132.

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