Effect of bax, bcl-2 and bcl-xL on regulating apoptosis in tissues of normal liver and hepatocellular carcinoma

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AIM: To investigate the expression of bax, bcl-2 and bcl-xL mRNA in the tissues of normal liver and hepatocellular carcinoma (HCC), and analyze the relationship between the expression of bax, bcl-2 and bcl-xL mRNA and clinical parameters of HCC patients.

METHODS: The expression of bax, bcl-2 and bcl-xL mRNA of normal liver and HCC was measured by Northern blot. Statistical analyses were made by t test and correlation analysis.

RESULTS: A very low mRNA level was indicated at bax, bcl-2 and bcl-xL in the HCC tissues in contrast to the tissues of normal liver by Northern blot analysis. The analyses of mRNA level revealed that HCC tissues exhibited a mean 7.6-fold decrease in bax, 4.2-fold in bcl-2 and 3.5-fold in bcl-xL in comparison with normal control tissues, respectively. Positive correlation was found between bax and bcl-xL (r = 0.7061, P < 0.01). There was no significance between the mRNA expression of these three genes and age, gender, tumor differentiation and tumor stage of HCC patients.

CONCLUSION: The results are consistent with the fact that apoptosis rarely occurs in normal livers but increases in HCC, indicating that bcl-2 and bcl-xL may play a very important role in regulating the apoptosis of normal liver and HCC.

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INTRODUCTION

Apoptosis is a highly regulated form of programmed cell death defined by distinct morphological and biochemical features. Programmed cell death is involved in a type of cell death, in which the cell actively uses a genetically controlled program to cause its own demise during the tissue remodeling of embryogenesis[1]. Apoptosis is a key mechanism causing cell death and organ diseases, failure of apoptosis is now understood to contribute to the development of human malignancies[2-5].

The bcl-2 family is the best characterized group of apoptosis-mediating factors, which include bcl-2, mcl-1, bcl-x, bax, bak, and several others. Although its members share close structural homologies, their biologic functions differentiate into apoptosis-promoting (bax, bak, bcl-xL) or apoptosis-inhibiting (bcl-2, mcl-1, bcl-xL) properties[6,7]. The bcl-2 related genes regulate cell death and are considered to correlate with the pathogenesis and progression of cancers[7-10]. Since the relationship between bcl-2 family and HCC is still unclear, we investigated the expression of the three genes in HCC and normal controls and evaluated the mediating action in HCC and the relationship between the genes and clinical parameters as well.

MATERIALS AND METHODS

Patients

Ten cases of (4 women, 6 men) normal liver tissues were obtained. The median age in the control group was 57 years, with a range of 39-75 years. HCC tissues were obtained from 21 patients (9 women, 12 men) undergoing surgery for HCC. The median age of the HCC patients was 64 years (a range of 33-76 years). According to the TNM classification of the International Union against Cancer, there were 6 patients with stage I, 13 with stage II, and 2 with stage IV disease. Tissues destined for RNA extraction were frozen in the operating room in liquid nitrogen immediately on surgical removal and maintained at -80°C until use.

Northern blot analysis

Total RNA was extracted by the guanidinium isothiocyanate method, fractionated on 1.2% agarose, 1.8 mol·L formaldehyde gels, and stained with ethidium bromide for verification of RNA integrity and loading equivalence[11]. The RNA was electrotransferred to Nylon membranes (GeneScreen; DuPont, Boston, Massachusetts, USA) and cross linked by UV irradiation. The filters were then prehybridized, hybridized, and washed under conditions appropriate for digoxigenin labelled antisense riboprobes (bcl-2 and bax) or the 32P labelled antisense riboprobe (bcl-xL) and cDNA probe (7S) as previously described[12,13].

In the case of the digoxigenin-labelled bcl-2 and bax cRNA probes, the filters were prehybridized and hybridized overnight at 68°C (bcl-2) or 65°C (bax) in a buffer containing 50% formamide, 2×SSC (1×SSC is 0.15M NaCl/0.015 M sodium citrate buffer) (bcl-2) or 5×SSC (bax), 2% blocking reagent (Boehringer Mannheim, Mannheim, Germany), 0.1% N-lauroylsarcosine, and 0.02% sodium dodecyl sulphate (SDS). The filters were then washed in 2×SSC/0.1% SDS at room temperature, followed by three 15 minute washings at 68°C (bcl-2) or 65°C (bax) in 0.065× SSC/0.065% SDS/35% formamide (bcl-2) or 0.1×SSC/0.1% SDS (bax). The filters were then incubated in a blocking buffer (1% blocking reagent in 100 mmol·L maleic acid/150 mmol·L NaCl, pH 7.5) for 30 minutes, and in blocking buffer containing anti-digoxigenin alkaline phosphatase antibody (1:20 000; Boehringer...
Mannheim, 1989) for 30 minutes, washed three times with maleic acid buffer for 15 minutes, and incubated with 25 mmol/L CDP-Star (Boehringer Mannheim). The membranes were then exposed to X-ray films.

For the ³²P labelled antisense riboprobe (bcl-2), the blots were prehybridized for 6 hours in 50% formamide, 0.5% sodium dodecyl sulfate (SDS), 5×SSC (sodium chloride/sodium citrate buffer), 5×Denhardt’s solution (1×Denhardt’s solution=0.02% ficol, 0.02% polyvinylpyrrolidone, and 0.02% bovine serum albumin), 25 mg/L salmon sperm DNA, and 50 mmol/L sodium phosphate buffer (pH 6.5). The blots were then hybridized for 18 hours at 65 °C in the presence of 1×10⁶ cp/ml labeled antisense riboprobe, washed twice at 65 °C in a solution containing 1×SSPE (150 mmol/L NaCl, 10 mmol/L NaH₂PO₄, and 1 mmol/L EDTA) and 0.5% SDS, and twice at 65 °C in a solution containing 0.1×SSPE and 0.5% SDS.

In the case of the 7S cDNA probe, blots were prehybridized for eight hours at 42 °C in a buffer which contained 50% formamide, 1% SDS, 0.75 mol/L NaCl, 5 mmol/L EDTA, 5×Denhardt’s solution, 100 mg/L salmon sperm DNA, 10% dextran sulfate, and 50 mmol/L phosphate buffer (pH 7.4). The hybridization was carried out at 42 °C for 18 hours by adding the ³²P labelled cDNA probe (1×10⁶ cpm/ml). The blots were rinsed twice in 2×SSC at room temperature and washed three times at 55 °C in 0.2×SSC/2% SDS under conditions appropriate for cDNA probes. Blots were then exposed at -80 °C to Fuji X-ray films with intensifying screens (DuPont).

For statistical analysis of the Northern blot results, the intensity of the radiographic bands was quantified by laser densitometry (Bio-Rad 620; Richmond, California, USA). The ratio between the bax, bcl-2 or bcl-xL and the corresponding 7S signal was calculated in each sample.

Statistical analysis
The data were expressed as median and range. Statistical analyses were carried out using the SPLM software (Statistical Department of Fourth Military Medical University). For statistical analysis, the t test and correlation analysis were used. Significance was defined as P<0.05.

RESULTS
Northern blot analysis
Northern blot analysis was carried out to determine bax, bcl-2 and bcl-xL mRNA expression in the normal and the cancerous liver. In contrast, hybridization signals of mRNA of three genes were higher in normal liver tissue as compared with the signals in cancerous samples (Figure 1). A low mRNA level of bax, bcl-2 and bcl-xL mRNA was almost present in all HCC tissue samples. In some HCC tissues the expression levels for all three genes were very faint and were only visible on the original autoradiographs. None of the normal or cancer samples showed any aberrant bax, bcl-2 or bcl-xL mRNA transcripts, and bax and bcl-2 mRNA were reduced in 100% of cancer samples, and bcl-xL mRNA was reduced in 95% of cancer samples in contrast with the normal controls. Densitometric analysis of the Northern blots indicated that the bax and bcl-2 mRNA levels in all cancer samples were 7.6- and 5.4-fold (P=0.0002; P=0.0087) higher than those in the matched control samples; bcl-xL mRNA levels in all cancer samples were 3.5-fold lower (P=0.0002) than those in the normal samples (Table 1). The expression of bax and bcl-xL mRNA was positively correlated in HCC (r=0.7061, P<0.01), but there was no correlation between bax and bcl-2 or bcl-xL and the corresponding 7S signal (r=0.1637, r=0.4830).

Statistical analysis revealed no relationship between bax, bcl-2 or bcl-xL expression and these parameters.

DISCUSSION
Apoptosis is a central regulator of tissue homeostasis. It contributes to the elimination of damaged cells in normal tissues and balances the appropriate cell number under the circumstances of physiologic cell proliferation and tissue repair.
In the past few years, scientific interest has focused on the process of apoptosis. Like cell replication, apoptosis is controlled by the network of positive and negative growth signals. Based on the currently prevailing views, it is assumed that malignant cells should be incapable of apoptosis and/or not responsive to death signals, thereby allowing unrestrained growth of cancer. Recently, more and more studies indicated that apoptosis is of importance in the growth and development of many tumors, but the pattern of apoptosis varies in different tumors. In contrast with normal tissues, there is often reduction of apoptosis in most cancerous tissues, but hepatic cancer is different. HCC is one of the most common and aggressive tumors in the world today, and little is known about the cellular pathogenesis. The apoptosis is rare in normal liver tissues (there is only 2-4 apoptotic cells per 10,000 hepatic or biliary cells), while the HCC tissues have higher rates of apoptosis. These findings indicate that the apoptosis-related genes are expressed in various frequencies in different cancers, and a general pattern of activation or inactivation of these genes in malignant tumors cannot be defined. Therefore, the function of apoptotic genes in different human cancers must be evaluated individually.

In this study, we analysed the concomitant expression of bax, bcl-2 and bcl-XL in the HCC tissues and normal liver. These genes belong to the same family of apoptotic genes. Although the structures are similar, they exert opposite effects on apoptosis. bcl-2 and bcl-XL inhibit apoptosis and contribute to cell survival and the resistance of cells against damaging influences. In contrast, bax, which is considered to be a central regulator of apoptosis, is a promoter of programmed cell death. The relationship between bcl-2 related genes and HCC is still unclear.

By the analysis of 21 HCC patients, reduced bax and bcl-2 signals were present in all samples and reduced bcl-XL signals were present in 95% of the cancer samples. The low expression of bcl-2 and bcl-XL is consistent with enhanced apoptosis in the HCC, but the low expression of bax does not account for this phenomenon. The findings revealed that anti-apoptotic genes, but not apoptosis-promoting genes, might play a more important role in regulating the apoptosis of normal liver and HCC. There was no correlation between bax, bcl-2 and bcl-XL and age, gender, differentiation or stage of tumor in HCC patients. We can not give a reasonable explanation for the finding that the expression of bax and bcl-XL was positively correlated in HCC. We concluded that anti-apoptotic genes do not influence the differentiation and development of HCC, but these genes can increase the rate of apoptosis in HCC by reducing their expression or changing the ratio with other genes. The influencing factors of apoptosis increased in HCC are not known, which may include 1) inherent metabolic process of tumor tissues; 2) hypoxia of tumor tissues; 3) some cytokines such as tumor necrosis factor; 4) attack of CTL, etc.

The low expression of anti-apoptotic genes, which increase apoptosis in HCC, may have negative impact on growth of tumors as a homeostasis mechanism that inhibits cell group with extensive growth, and this effect can delay the development of tumors. But it may also be a selective pressure, which removes the aging tumor cells or some tumor cells with phenotype similar to normal cells and selects more aggressive and prosperous clone of tumor cells, accelerating the development of tumors. Further studies on the regulating action of apoptotic genes will deepen the understanding about the growth and development of HCC and offer valuable information to genetic therapy of HCC, thus enhancing the sensibility to radiotherapy and chemotherapy.

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