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SHORT COMMUNICATION

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Immunohistochemical study of pro-apoptotic factors Bax, Fas and CPP32 in urinary bladder cancer: prognostic implications

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Abstract Apoptosis is a process which can retard the progress of malignant tumours. In the present study we investigated the immunohistochemical expression of three pro-apoptotic proteins (Bax, Fas receptor and caspase 3 – CPP32) as well as the apoptotic index (TUNEL Index) in 53 urothelial carcinomas of the urinary bladder. We examined all possible relationships between these factors and clinicopathological variables, as well as the patients' disease free survival. All three markers, and in particular the Fas receptor, were detectable in a remarkable proportion of specimens, however, none of the markers examined was associated with any clinicopathological parameter. In multivariate statistical analysis, Bax emerged as an independent predictor of a favourable prognosis. It is noteworthy that none of the pro-apoptotic markers examined was directly linked with the apoptotic rate of the malignant cells.

Keywords Bladder · Carcinoma · Bax · Fas · CPP32

Introduction

Tumour growth is the result of a disturbance in the balance between cell proliferation rate and apoptosis. The latter process occurs in malignant neoplasms, often resulting in a marked retardation in their growth.

The Bcl-2 family of proteins regulate the cellular commitment to survive or die when challenged with various apoptotic stimuli [12]. Among these proteins, Bax is a primary-response gene for wild-type (w-t) p53 [11] and accelerates apoptosis by antagonizing the apoptosis repressor Bcl-2. Particularly in bilharzial related urothelial carcinoma (UC) of the bladder, Bax immunoexpression has recently been proposed as an independent prognostic factor [3].

Apoptosis is implemented by a death machinery, the executionary arm of which is a family of cysteine proteases called caspases. When activated, these cleave key substrates in the cell to bring about an apoptotic morphology [8]. Caspase 3 (CPP32) has been postulated to be best correlated with the initiation of apoptosis because of its location as the most downstream enzyme in the apoptosis-inducing protease pathway. Proteolytic activation of CPP32 is blocked by overexpression of Bcl-2 but CPP32 is able to reverse the function of Bcl-2 by cleaving it to a truncated, pro-apoptotic form. Generally, CPP32 is believed to be cleaved by other, upstream activators in receptor-mediated apoptosis, such as ligation of the Fas death-inducing membrane receptors [5]. The interaction of Fas and Fas ligand plays an important role in cytotoxic T-lymphocyte-mediated and natural killer cell-mediated apoptosis against tumour cells [6]. Apart from the Bcl-2 family-dependent apoptotic pathway, which occurs in various kinds of malignant cells, apoptosis triggered by Fas (Apo-1/CD95) is not blocked by over-expression of Bcl-2; Fas directly induces caspase activation [12]. Fas and Bax operate mechanistically via distinct intracellular pathways to elicit the apoptotic effect in response to differential signals.

In the present study, we focus on the immunohistochemical expression of Bax, Fas and CPP32 in a well documented series of 53 UCs of the urinary bladder and search for any association with the degree of apoptosis as assessed by the TUNEL Index (TI) as well as with data available from previous investigations [7]; i.e., p53 and Bcl-2 protein's immunostatus, proliferation immunomarker Ki-67, flow cytometric DNA content (diploid or aneuploid) and the patient's clinicopathological data [i.e. gender, age, low or high grade in

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non-invasive UCs according to the new WHO scheme [2], local stage (pT)] and disease-free survival (median follow-up period: 46 months, range: 6–73 months). Grading of the invasive component was also performed according to the previous version of the WHO classification.

Material and methods

No in situ UCs or papillary neoplasms of low malignant potential were included in the study. Of the 53 UCs, eight were non-invasive papillary (pTa), 27 were papillary with an associated invasive component and 18 were invasive without an accompanying papillary component. With regard to the level of infiltration, 22 were pT_1, 10 were pT_2 and 13 pT_3. Paraffin sections were stained by standard immunohistochemistry the technical details of which have been reported previously [7]: The following antibodies from Santa Cruz Biotech (Calif., USA), were applied: mouse monoclonal B-9 (epitope corresponding to aminoacids 1–171), goat polyclonal N-19 (epitope mapping at the amino terminus) and rabbit polyclonal C-20 (epitope mapping at the carboxy terminus) for Bax, CPP32 and Fas receptor, respectively. Categorization of immunostaining for all three markers was made semiquantitatively by microscopic observation according to the following, statistically appropriate cut-off points: expression in $< 10\%$ of malignant cells = negativity status, 10–29% = low positivity status and $\geq 30\%$ = high positivity status. Apoptotic cells were detected by labeling of fragmented DNA (TUNEL, in situ cell detection labelling kit, POD, Roche Molecular Biochemica, Mannheim, Germany) as previously described [10], in combination with conventional apoptotic morphological criteria. Apoptotic cells were counted by an image analysis system with an appropriate software package (Sigma Scan Pro, Version 5.0: SPSS Science, Erkrath, Germany). Pearson's χ^2 tests were applied for the statistical analysis of all categorical data while concordance between Bax, Fax and CPP32 was assessed by McNemar's χ^2 test (SPSS, V.8, Chicago, IL). Disease-free survival curves were plotted by both univariate (log-rank test) and multivariate (Cox proportional hazard model) analysis.

Results

Apoptotic cells were noticeable in all examined specimens (mean apoptotic index: 7.45%; median: 4%; maximum 23.2%; minimum 0.22%). The three apoptosis-related proteins, Bax, Fas and CPP32, were positively detected in a considerable proportion of UCs [54.7% (29/53), 81.1% (43/53) and 52.8% (28/53), respectively]. Fas immunostaining demonstrated a cytoplasmic pattern (Fig. 1). The other two apoptosis-related immunomarkers (Bax and CPP32) also demonstrated cytoplasmic staining patterns, while TUNEL labelling was detected in the nuclei of apoptotic cells (Fig. 2). Tumour adjacent, morphologically normal urothelium, when observable, was practically negative for Fas staining and only focally positive for Bax (Fig. 3) and CPP32, while a minimal presence of TUNEL-positive cells was occasionally noticeable. No statistical association could be detected between any of the above proteins and any clinicopathological variable (including the presence of a papillary component, level of infiltration and grade both of the non-invasive and invasive lesions), expression of p53 or Bcl-2 protein, degree of proliferation, DNA content, or apoptotic index. McNemar's test showed that the incidence of immunopositivity was not the same either for Fas and Bax or for Fas and CPP32. Fas immunopositivity incidence was found to be 25.8%

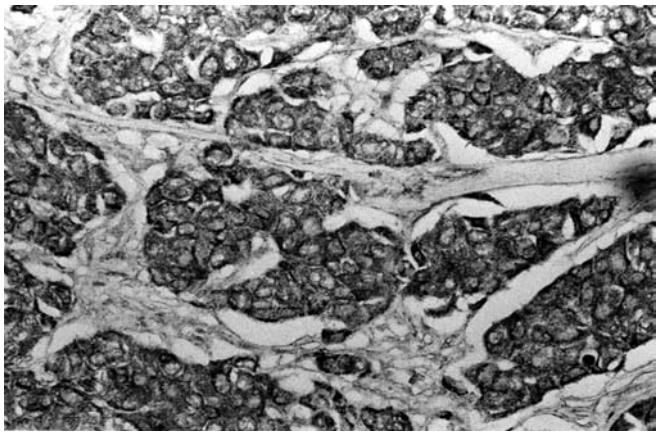

Fig. 1. Fas cytoplasmic immunopositivity in an invasive UC (immunoperoxidase stain, ×300)

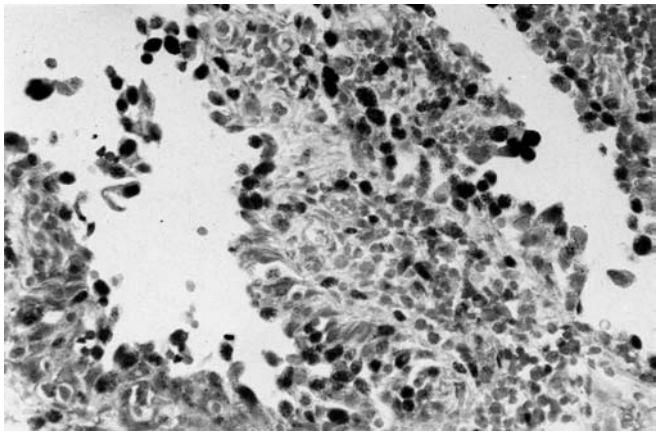

Fig. 2. Nuclear staining in a considerable number of apoptotic cancer cells (TUNEL, ×300)

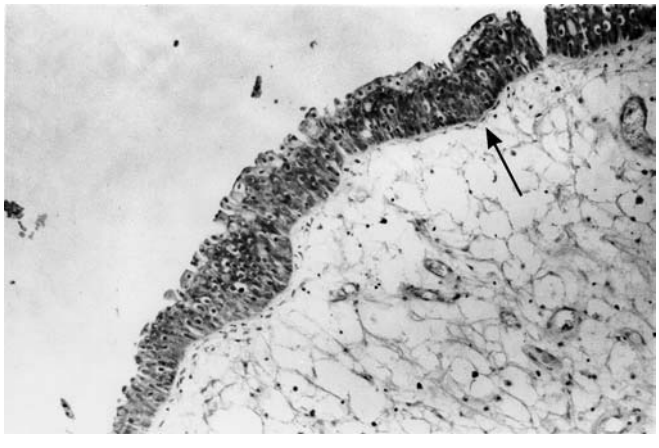

Fig. 3. Focal bax immunoreactivity (*arrow*) in normal urothelium of a patient with UC (immunoperoxidase stain, ×200)

higher than Bax immunopositivity (95% CI: 12.8%–38.7%) ($P < 0.001$) and the former incidence was found to be 28.7% higher than CPP32 immunopositivity (95% CI: 15%–42.4%) ($P < 0.001$).

Twenty one of the patients in this study relapsed during the follow-up period. Kaplan-Meier analysis revealed that patients with Fas-negative UCs tended to have a longer disease-free survival rate than the patients with Fas-positive UCs ($P = 0.082$). With regard to multivariate disease-free survival analysis, when stage and grade were included in the Cox regression model, Bax immunopositivity was an independent, favourable prognostic factor ($P = 0.008$) (Fig. 4). Its statistical significance was only less than that of stage ($P = 0.003$). When grade was excluded from the model, Bax and stage retained their significance but this time high TI emerged as a significant, favourable predictor ($P = 0.036$). When the influence of neither grade nor stage was considered, the borderline negative influence of Fas positivity reached statistical significance ($P = 0.020$). Bax and TI were again statistically significant variables of a favourable prognosis.

Discussion

Despite their reported biological interrelationships, all three pro-apoptotic factors were detected independently of each other. Among them, the Fas receptor was likely to participate in apoptosis more often than Bax or CPP32. Further study of the Fas receptor interaction with its ligand is needed so that the Fas system role in bladder cancer can be clarified [4]. The lack of relation of CPP32 expression with degree of apoptosis has also been reported in other tumours and may be due to immunohistochemistry, which cannot discriminate between the active and inactive forms of the caspases. By immunohistochemistry, the presence of the pro-apoptotic proteins was shown and at least as far as Bax immunopositivity is concerned, its incidence was almost identical to that of another recent study [9]; however, the functional role of these three proteins cannot be assessed. On the other hand, it is true that TUNEL can by no means exclusively and/or selectively detect apoptotic cells, although when nuclear morphology is correlated with TUNEL results, this technique can be considered one of the best in situ methods of detecting apoptosis. The control of DNA fragmentation, as measured by the TUNEL assay, appears to be rather complex since none of the assessed apoptotic proteins could be directly linked with the apoptotic index of tumour cells.

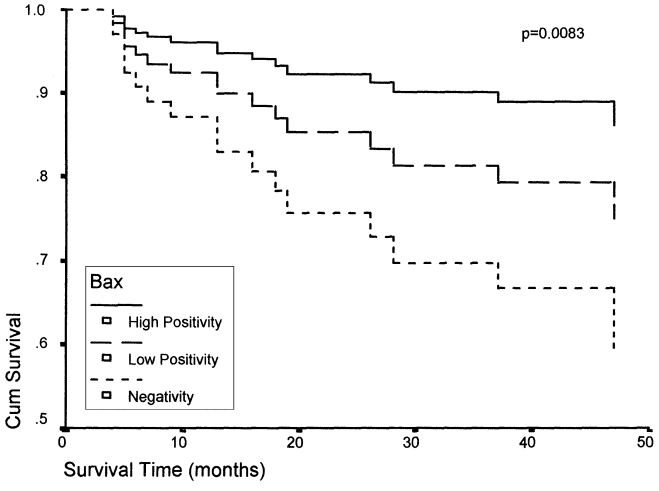

Fig. 4. The positive prognostic value of Bax immunoreactivity in the examined patients (from multivariate statistical analysis)

Two pro-apoptotic factors, Bax and Fas, were found to have opposite effects on a patient's disease-free survival, while CPP32 had no influence. An increased apoptotic rate does not permit cancer cells with accumulated genetic damage, and thus potentially aggressive biological behaviour, to grow. This may be linked to the favourable influence of high TI and Bax immunopositivity. Actually, high Bax expression has already been correlated with a favourable prognosis in other tumours. The favourable prognostic influence of Bax positivity in UCs is probably in parallel with the similar effect reported for the Bcl-2/Bax protein ratio being less than 1 [1, 13]. In the present series, however, Bcl-2 did not seem to have a critical role in determining relapse-free survival [7].

In contrast to Bax, Fas immunoreactivity was an unfavourable prognostic factor although, theoretically, Fas transduces a death signal to the cell on which it is expressed and so exerts an apoptotic function like Bax. Nevertheless, Fas-positive UCs often demonstrated diffuse cytoplasmic staining without clear cell surface expression (Fig. 1). The detection of the Fas receptor may not correlate with its biological function. In fact, the retention of Fas protein within the cytoplasm may represent a mechanism by which malignant cells evade Fas-mediated apoptosis. The observed negative prognostic influence of Fas cytoplasmic staining may actually be connected to apoptotic resistance. In a minority of our Fas-positive UCs, neoplastic cells were able to translocate the protein to the cell membrane. This Fas expression is more likely to predict susceptibility to killing but the number of such positive cases was too small for proper statistical analysis.

In conclusion, the pro-apoptotic immunohistochemical markers examined, of which at least two (i.e. Fas and CPP32) have not been adequately investigated in bladder cancer, were not directly linked to the apoptotic index of malignant cells. With regard to disease-free survival, Bax protein emerges as a promising, favourable indicator.

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