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Bcl-2 immunoreactivity in prostate tumorigenesis in relation to prostatic intraepithelial neoplasia, grade, hormonal status, metastatic growth and survival

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Abstract The Bcl-2 protein prolongs cell survival by overriding apoptosis. To explore the role of Bcl-2 in prostate tumorigenesis, immunoreactivity for Bcl-2 was examined in untreated and androgen-deprived tumours and lymph node metastasis. Following the transurethral resection, 150 untreated patients were maintained under surveillance until death or for a minimum of 11 years, and castration was performed at symptomatic progression. The Bcl-2 index (BI) was defined as the percentage of immunoreactive cells in a tumour. The mean BI was 12 in the untreated tumours, and BI was significantly higher in high-grade tumours, mean BI 17, than in low-grade tumours, mean BI 6. There was no correlation between BI and stage or metastatic disease, nor did BI predict cancer-specific survival. In 16 androgen-deprived, but non-relapsed tumours, the mean BI was 54, at a mean time of 22 months after castration, indicating a permanent increase of Bcl-2 protein expression after androgen withdrawal. In six patients, tissues from the prostate tumour and obturator lymph node metastasis were available. Four primary tumours immunostained for Bcl-2, but only one metastasis stained. Foci of high-grade prostatic intraepithelial neoplasia (PIN) were present in 44 of the 150 untreated tumours. All PIN foci were intensely immunoreactive for Bcl-2, and mean BI was 79, suggesting that Bcl-2 protein expression is associated with early prostate tumorigenesis.

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Introduction

The number of patients diagnosed with prostate cancer increases each year [11, 18], and the prognosis and optimal therapy in each stage of the disease remain controversial [27]. There is consequently an urgent need for better predictors of progression and growth potential in prostate tumours [6]. Cell net growth is determined by the balance between proliferation and apoptosis. In prostate cancer, the proliferation rate is very low [1] and there are reasons to believe that the rate of apoptosis in the prostate may be critical for tumorigenesis. Bcl-2 is a key regulator of apoptosis, prolonging cell survival by inhibition of apoptosis [34] induced by many different stimuli [8, 12, 26]. In experimental systems, Bcl-2 overexpression was found to prolong cell survival in transgenic mice [19] and to inhibit apoptosis induced by DNA-damaging agents including many chemotherapeutics [26]. Bcl-2 protein has been demonstrated in stem cells with prolonged survival in normal tissues, e.g. bone marrow progenitor cells, and basal cell layer in epithelia of the GI tract, breast and prostate [13]. Furthermore, it has been demonstrated in dysplastic precursor lesions to cancer, in manifest cancers of the breast [17] and colon [3, 31], and in foci of gastric epithelial dysplasia [16].

In the human prostate, Bcl-2 protein has been shown to be present in all foetal cells, in the basal cell layer of the normal prostate, in foci of prostatic intraepithelial lesions (PIN) and in 30–60% of localized prostate tumours [7, 20]. In the rat prostate, *bcl-2* expression has been shown to be induced in the short term after castration [20] and *bcl-2*-transfected LNCAP tumours grown in nude mice were refractory to castration therapy [25]. In human prostate tumours, Bcl-2 staining

was increased both 1 week after castration [36] and in relapsed tumours [7, 20]. Moreover, tumours Bcl-2 immunoreactive prior to castration showed no increase in the rate of apoptosis 1 week after therapy [36]. All these data indicate that Bcl-2 protein expression may be of importance in prostate tumorigenesis, and that Bcl-2 immunoreactivity could potentially be a predictive marker for outcome. However, the predictive value of Bcl-2 staining in prostate cancer is as yet unexplored.

Materials and methods

Tissue samples

Specimens from transurethral resections (TURs) of prostate tumours, formalin-fixed and paraffin-embedded, were obtained from the pathology files of the Departments of Pathology, at the Central Hospital, Västerås, and the University Hospital, Umeå, Sweden.

In the first group, 150 tumours randomly chosen from a series of 186 prostate cancers consecutively diagnosed at the Central Hospital in Västerås between 1975 and 1983 were examined. These patients had not received any cancer therapy prior to TUR. Staging was performed at the time of surgery, local tumour stage was determined by digital rectal examination according to the 1978 UICC classification [33] and radionuclide bone scan was performed for M-staging. The tumours were re-examined and graded by one pathologist according to the WHO classification [22] (Table 1). High-grade intraepithelial neoplasia was classified as described by McNeal and Bostwick [21]. After TUR, the patients were followed with surveillance for a minimum of 11 years or until death. Castration was performed if the patient had symptomatic progression. Nineteen patients (13%) were still alive in May 1994. Mean overall survival was 67 months, 64 patients (43%) died from prostate cancer and 67 patients (44%) died from other causes. The cause of death was determined by examination of the patient records.

In the second group, tissues from six primary prostate tumours and the corresponding lymph node metastasis were available for examination. Prostate tissue was obtained through TUR in four patients and needle biopsies in two patients. Lymph node tissue was obtained through staging pelvic lymphadenectomy.

In the third group, 17 androgen-deprived tumours were investigated, and the mean time between castration and TUR was 22 months. The indication for TUR was micturition problems in all cases. Areas of persisting cancer were present in all specimens. To determine if relapse had occurred, the serum prostate-specific antigen (PSA) level was measured and clinical status was evaluated at the time of TUR. Relapse was defined as recurrent symptoms of generalized disease, e.g. bone pain, weight loss or serum PSA levels above 30 ng/ml (Table 2).

Immunohistochemistry

Representative areas of the cancer were identified on haematoxylin/eosin-stained slides. Adjacent 4-μm-thick sections were deparaffinated and rehydrated according to standard procedures, washed with phosphate-buffered saline (PBS) solution and heated in a microwave owen at 600 W for 20 min in 0.01 M citrate buffer, pH 6, as described earlier [29]. The sections were pretreated with 0.1% bovine serum albumin in PBS and incubated overnight with the primary monoclonal anti-Bcl-2 oncoprotein (clone 124, Dako, Denmark) diluted to 1/100. The biotin streptavidin method was used (Supersensitive Multi-link kit, BioGenex San Ramon, CA, USA) according to the manufacturer's instructions. Briefly, the sections were incubated in chronological order with a biotinylated Multi-link secondary antibody for 20 min, washed for 10 min in PBS, incubated with alkaline phosphatase for 20 min and washed again for 10 min in PBS. The sections were then developed for 20 min using the chromogen fast red, rinsed briefly in water and lightly counterstained with Meyer's haematoxylin solution and mounted. Lymphoid tissue from tonsillectomies was used as positive controls. Substitution of the anti-Bcl-2 antibody with normal mouse serum was used as negative control. The immunoreactivity was evaluated in 500 cells in each section. Cells for evaluation were selected with the aid of a grid moved at random across the section. Bcl-2 index (BI) was defined as the number of immunoreactive cells of the total number of evaluated cells in a tumour. All sections were evaluated in a blinded procedure without knowledge of any patient data.

Table 1 Clinical characteristics and Bcl-2 immunoreactivity in untreated prostate tumours, obtained through transurethral resection (*PIN* prostatic intraepithelial lesion)

	No.	Mean Bcl-2 index[a] (SE)
Total No. of patients	150	12 (1.7)
Tumour stage[b]		
T0 focal	25	9 (3.6)
T0 diffuse	30	8 (2.9)
T1	28	13 (3.9)
T2	47	15 (3.3)
T3	10	15 (6.2)
T4	6	3 (2.0)
Tx	4	28 (22)
Metastasis[c]		
M0	75	13 (2.6)
M1	17	9 (4.5)
Mx	58	11 (2.7)
Grade[d]		
G1	36	6 (2.3)
G2	74	12 (2.5)
G3	40	17 (3.6)
PIN	44	79 (1.7)
Mean age 74 years		
Age < 74 years	81	12 (2.3)
Age > 74 years	69	11 (2.5)

[a] Bcl-2 index was defined as the percentage of immunoreactive cells in a tumour
[b] Local tumour stage according to the 1978 UICC classification [33]
[c] Presence of metastasis determined by bone scan
[d] *G1* highly differentiated, *G2* moderately differentiated, *G3* poorly differentiated according to WHO [22]

Statistics

To test the correlations the Spearman rank sum test was applied. The paired *t*-test was used to test the difference in paired observations and the *t*-test for independent samples was used for comparing means for different groups. Cancer-specific survival was estimated using the Kaplan-Meier method, and comparisons between groups were made by the log rank test. A P value of less than 0.05 was considered significant in all tests applied [4, 23].

Results

In the benign prostate, Bcl-2 staining was consistently observed in the cytoplasm of cells in the basal layer in

the ducts and acini, but staining was always absent in luminal cells. Infiltrating lymphocytes were always intensely positive and served as an internal control. The immunoreactivity for Bcl-2 was heterogeneous in the tumours, and intensely stained areas and entirely negative areas were observed adjacent to each other. There was a broad variation in staining intensity between the tumours, but in general positive tumour cells stained rather weakly compared to lymphocytes and basal epithelial cells (Fig. 1a–c). No specific pattern of tumour growth was observed in the Bcl-2-positive areas. Bcl-2 protein expression was present in 92/150 (61%) of the untreated tumours, with a mean BI of 12 (range 0.1–95). There was a significant correlation between BI and WHO grade ($r_s = 0.22$, $P = 0.007$), and the mean BI was 6 for G1, 12 for G2 and 17 for G3 tumours (significant difference only between G1 and G3, $P = 0.02$). There was no correlation between BI and local tumour stage, presence of metastasis or patient age (Table 1). To explore data, different cutoff levels for BI positivity were systematically examined by Kaplan-Meier analysis for cancer-specific survival. There was a slight association between longer cancer-specific survival and low BI, but this did not attain significance at any cutoff level.

Foci of high-grade PIN could be identified in 44/150 (29%) tumours. In these foci, Bcl-2 staining was intense in all cell layers including the luminal cells (Figs. 1d, 2b). The mean BI for PIN areas was 79. This was significantly higher than mean BI in manifest cancer areas, 79 vs 12 ($P < 0.0001$). There was no relation between the BI in the PIN areas and grade, stage or presence of metastasis. Furthermore, no predictive value for cancer-specific survival was found for BI in PIN. In the paired specimens four out of six of the primary tumours stained for Bcl-2; one of these stained only in foci of PIN, but only one out of six of the lymph node metastasis stained (Fig. 1e, f, Table 2).

Table 2 Bcl-2 immunoreactivity in primary prostate tumour and corresponding lymph node metastasis (*PIN* prostatic intraepithelial lesion)

Patient No.	Grade primary tumour[a]	Bcl-2 index[b]		
		Cancer areas in primary tumour	PIN foci in primary tumour	Lymph node metastasis
1	G2	40	100	0
2	G2	17	84	22
3	G2	59	Not present	0
4	G2	0	100	0
5	G2	0	Not present	0
6	G2	0	Not present	0

[a] Grade according to WHO [22]
[b] Bcl-2 index defined as the percentage of immunoreactive cells in a tumour

Table 3 Clinical features, Bcl-2 immunoreactivity and outcome of patients castrated prior to TUR

Patient No.	T stage[a]	M stage[b]	Grade[c]	Castration–TUR time (months)	Serum PSA	Status at time of TUR[d]	Bcl-2 index[e]	Observation/ survival (months after TUR)	Cause of death
1	T2	M0	G1	53	–	*n*	89	41	Alive
2	T0	M0	G2	10	2	*n*	31	41	Other cause
3	T2	M0	G2	11	3	*n*	96	28	Prostate cancer
4	Tx	M0	G2	7	7	*n*	43	8	Prostate cancer
5	T1	M0	G3	48	30	*n*	65	39	Alive
6	T1	M0	G3	23	5	*n*	0	14	Prostate cancer
7	T2	M0	G3	5	1	*n*	50	55	Alive
8	T3	M0	G3	22	1	*n*	60	6	Other cause
9	T4	M0	G3	83	16	*n*	39	22	Alive
10	T3	M1	G2	10	–	*n*	67	21	Other cause
11	T3	M1	G2	3	3	*n*	84	39	Prostate cancer
12	T3	M1	G2	16	2	*n*	23	32	Other cause
13	T3	M1	G2	9	13	*n*	80	5	Other cause
14	T3	M1	G2	6	138	*R*	40	13	Prostate cancer
15	T4	M1	G2	34	3	*n*	69	43	Alive
16	T2	M1	G3	19	5	*n*	30	58	Cause unknown
17	T3	M1	G3	14	12	*n*	54	39	Prostate cancer

[a] Local tumour stage according to the 1978 UICC classification [33]
[b] Presence of metastasis determined by bone scan
[c] G1 highly differentiated, G2 moderately differentiated, G3 poorly differentiated according to WHO [22]
[d] Relapse defined as recurrent symptoms of generalized disease, bone pain, etc., or PSA >30 ng/ml, *n* not relapsed, *R* relapsed
[e] Bcl-2 index was defined as the percentage of immunoreactive cells in a tumour

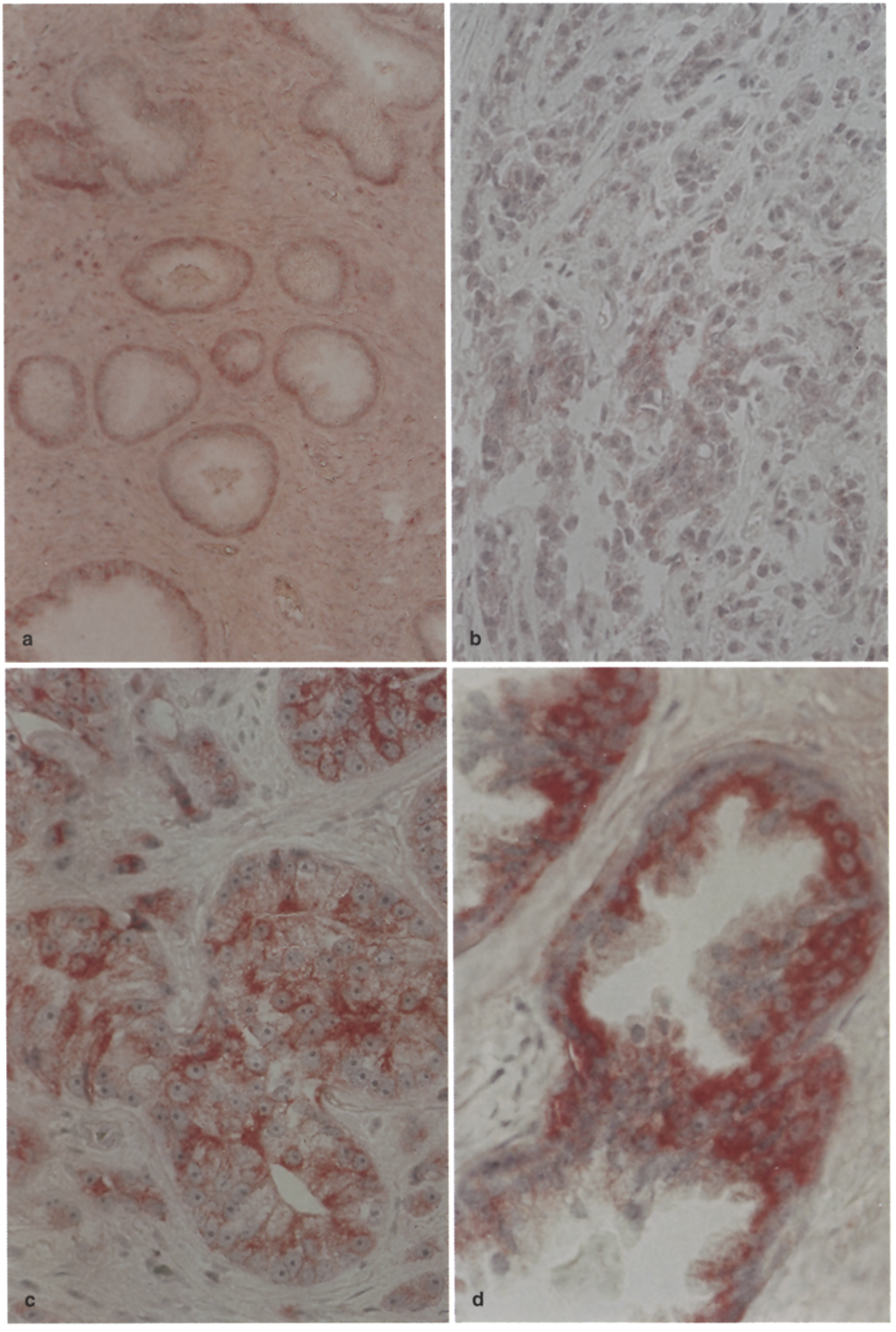

Fig. 1a–d

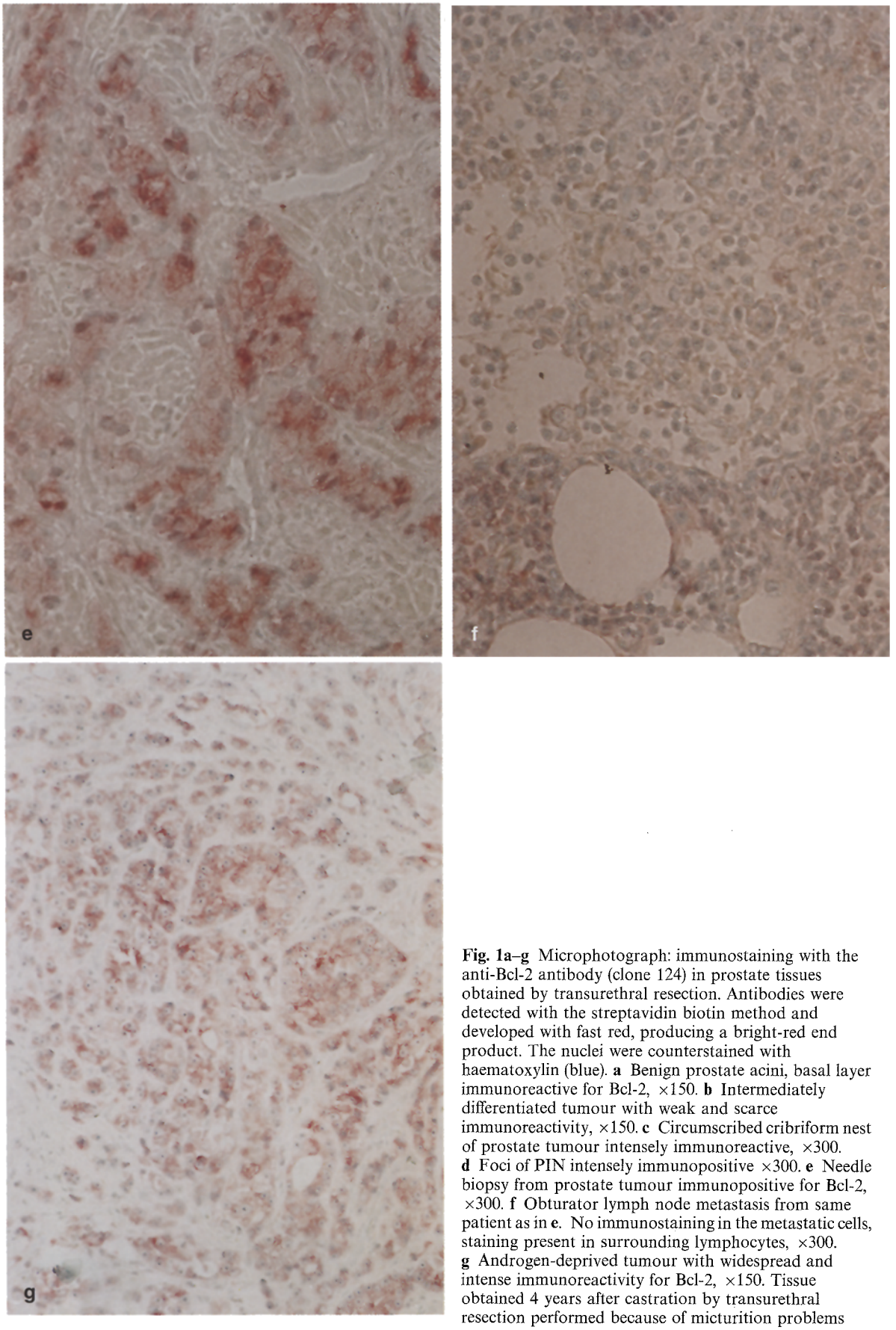

Fig. 1a–g Microphotograph: immunostaining with the anti-Bcl-2 antibody (clone 124) in prostate tissues obtained by transurethral resection. Antibodies were detected with the streptavidin biotin method and developed with fast red, producing a bright-red end product. The nuclei were counterstained with haematoxylin (blue). **a** Benign prostate acini, basal layer immunoreactive for Bcl-2, ×150. **b** Intermediately differentiated tumour with weak and scarce immunoreactivity, ×150. **c** Circumscribed cribriform nest of prostate tumour intensely immunoreactive, ×300. **d** Foci of PIN intensely immunopositive ×300. **e** Needle biopsy from prostate tumour immunopositive for Bcl-2, ×300. **f** Obturator lymph node metastasis from same patient as in **e**. No immunostaining in the metastatic cells, staining present in surrounding lymphocytes, ×300. **g** Androgen-deprived tumour with widespread and intense immunoreactivity for Bcl-2, ×150. Tissue obtained 4 years after castration by transurethral resection performed because of micturition problems

Bcl-2 reactivity was found in 16/17 (94%) of the androgen-deprived tumours, the mean BI in these tumours being significantly higher than in the untreated tumours, 54 vs 12 ($P < 0.001$) (Figs. 1g, 2c). Only one patient had a relapse at the time of TUR (serum PSA was 138 ng/ml, and the patient developed clinical symptoms shortly after the operation) and BI was 40 in this tumour. The remaining 16 patients had no symptoms of relapse at the time of TUR, and their mean serum PSA value was 7.3 ng/ml. Mean survival after TUR was 30 months (Table 3). No foci of PIN could be detected in these androgen-deprived tumours. There was no significant difference in mean BI between grades in the androgen-deprived group. In an analysis of untreated and androgen-deprived tumours grade by grade, mean BI was significantly higher in the androgen-treated group in G2 ($P = 0.001$) and G3 tumours ($P = 0.02$); no analysis of the G1 tumours was performed as there was only one G1, androgen-deprived tumour (Fig. 2d).

Discussion

In this study, strong Bcl-2 immunoreactivity was observed in all foci of high-grade PIN in untreated prostate tumours. This is in accordance with Colombel et al. [7], who found four out of four foci of PIN to be Bcl-2 positive. High-grade PIN has been indicated by several authors to be a precursor to prostate cancer [2, 21]. Bcl-2 protein expression may thus be involved in early prostate tumorigenesis, probably by prolonging cell survival, thereby rendering the PIN cells more vulnerable to other oncogenes, which could induce further steps in the tumorigenesis. In line with this, Bcl-2 staining is increased in other precursor lesions to cancer, such as adenomas of the colon [3, 31], foci of gastric epithelial dysplasia [16] and cancer in situ of the breast [17].

In manifest cancers, Bcl-2 immunoreactivity was less prominent than in the foci of PIN. Staining in the cancer areas was heterogeneous and our data seem to reconcile some apparently contradictory results in earlier studies. Colombel et al. [7] found 23 out of 37 (62%) tumours to be immunoreactive, but McDonnell et al. [20] showed Bcl-2 staining to be more than "rare and widely scattered" in only 6 out of 19 (32%) tumours in their series. Westin et al. [36] found Bcl-2 staining in 8 out of 18 (44%) tumours. The cutoff level for positivity is apparently important; however, collectively these studies suggest that only a small proportion of prostatic tumours have widespread staining. Bcl-2 protein expression did not seem to be a prerequisite for metastatic growth, as only one out of six lymph node metastases was positive. No predictive value for cancer-specific survival was found for Bcl-2 immunoreactivity.

Studies of Bcl-2 immunoreactivity in relation to histological grade and prognosis in other tumour forms have given conflicting results. Neuroblastomas demonstrated a significant correlation between Bcl-2 staining and poor differentiation [5]. Bcl-2 staining was elevated in differentiated thyroid cancer, but low in undifferentiated forms [24]. Higher Bcl-2 levels were found to be higher in normal melanocytes and common acquired naevi than in malignant melanomas [32]. One study in breast cancer showed a significant correlation between Bcl-2 staining and well-differentiated tumours [14], and several studies have shown Bcl-2 levels to be higher in tumours with oestrogen receptor positivity, known to be a favourable prognostic factor [14, 17, 30]. Both Joensuu et al. [14] and Silvestrini

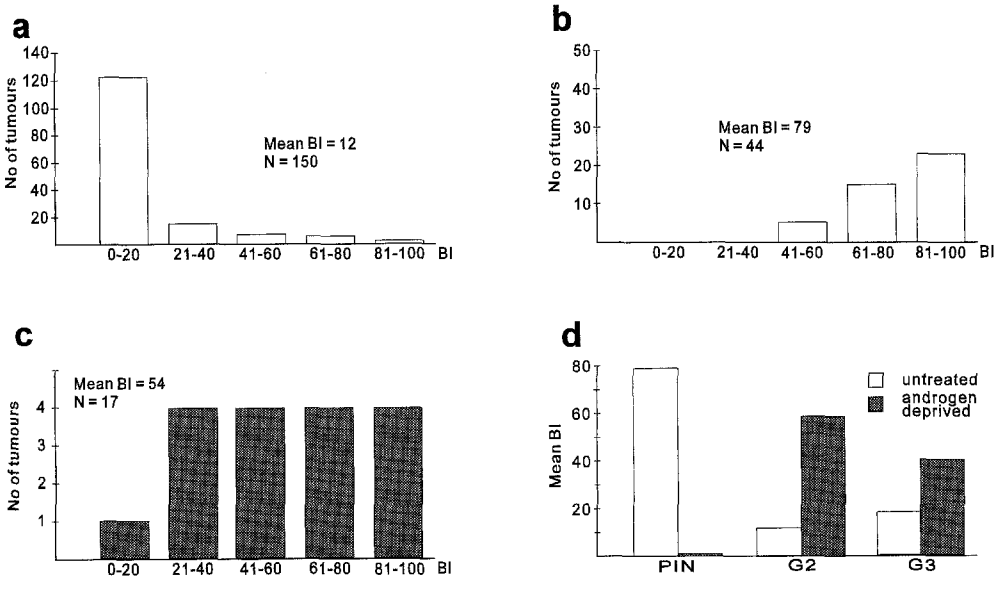

Fig. 2a–d Distribution of Bcl-2 immunoreactivity in prostate tumours. **a** Cancer areas in untreated tumours. Bcl-2 index (*BI*) defined as percentage of immunoreactive cells in a tumour. **b** Foci of PIN. Foci of prostatic intraepithelial neoplasia (*PIN*) demonstrated in 44/150 of the untreated tumours. **c** Androgen-deprived tumours. Castration performed a mean time of 22 months before TUR. **d** Influence of hormonal status. Mean Bcl-2 index in untreated and androgen-deprived prostate tumours compared grade by grade. Highly differentiated tumours (*G1*) not analyzed due to small number. *G2* indicates intermediately differentiated tumours, *G3* poorly differentiated tumours [22]

et al. [30] found a more *favourable* outcome for breast cancers with *high* levels of Bcl-2 immunoreactivity. Moreover, Viale et al. recently showed a significantly longer survival for Bcl-2-immunoreactive medullary thyroid carcinomas, Bcl-2 being the only factor that significantly predicted survival in a multivariate analysis [35]. This paradoxical influence of the apoptosis suppressing Bcl-2 protein on survival may not be surprising, since it is known that Bcl-2 interacts with antagonizing homologues (bax, bad, bak), proteins that promote apoptosis [26]. It has been proposed that it is the balance between these proteins, and not the Bcl-2 levels alone, that determines whether or not the cell will undergo apoptosis [8, 26]. In a study of metastatic breast cancer, the immunoreactivity for bax and Bcl-2 was highly correlated and low levels of bax were associated with short survival [15]. To date, it is unknown if these other apoptosis-regulating proteins are present in prostate tumours. Androgen deprivation has been shown to induce *bcl-2* expression in the rat ventral prostate in the short term [20] and *bcl-2*-transfected LNCAP tumours grown in nude mice were refractory to castration therapy [25]. In an earlier study, we showed that Bcl-2 immunoreactivity was increased in human prostate tumours in biopsies 1 week after castration [36]. In this study, androgen-deprived, non-relapsed tumours were immunoreactive for Bcl-2 at a mean time of 22 months after castration. Together these data suggest a rapid and permanent increase in the Bcl-2 protein expression in prostate cancer after androgen witdrawal before relapse. Likewise, Bcl-2 immunoreactivity was distinctly elevated in the follicular phase in both the normal breast [28] and endometrial gland [10], and declined rapidly afterwards in the luteal phase. These observations suggest a regulation of Bcl-2 protein expression by sex hormones in hormonally dependent tissues. However, the question whether Bcl-2 expressing prostate tumour cells are hormonally dependent or not seems to be complex. On one hand, PIN cells are considered to be androgen dependent and disappear after androgen ablation [9]. In line with this, PIN cells, with high levels of Bcl-2 in the hormonally intact tumours, were absent in the androgen-deprived tumours. Thus, Bcl-2-expressing cells in PIN lesions seem to be androgen dependent and susceptible to apoptosis. On the other hand, Bcl-2 immunoreactivity was distinctly higher in androgen-deprived tumours than in hormonally intact tumours, suggesting a selection or adaptation for Bcl-2 expression in androgen-deprived tumours. These apparently contradictory results suggest that the interaction between Bcl-2 and homologues such as bax determines the difference in androgen dependence in PIN and tumour cells. Further studies are needed to elucidate this issue.

In conclusion, Bcl-2 staining was scarce in cancer areas, and only one out of six lymph node metastases stained for Bcl-2. Our results indicate a permanent elevation of Bcl-2 levels in prostate tumours after castration. No significant predictive value for cancer-specific survival was found for Bcl-2 immunoreactivity. Elevated, widespread Bcl-2 immunoreactivity was observed in high-grade PIN, suggesting a role for Bcl-2 in early prostate tumorigenesis.

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