JOURNAL OF PATHOLOGY, VOL. 180: 275–282 (1996)

Bcl-2 EXPRESSION IS CORRELATED WITH A LOW APOPTOTIC INDEX AND ASSOCIATED WITH PROGESTERONE RECEPTOR IMMUNOREACTIVITY IN ENDOMETRIAL CARCINOMAS

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SUMMARY

A total of 103 endometrial carcinomas (endometrioid type), as well as 15 samples of normal (atrophic or proliferative phase) and 26 of hyperplastic endometrium, were immunohistochemically investigated for expression of Bcl-2, and oestrogen and progesterone receptors (ER and PR), and the results compared with findings for apoptosis and cell proliferation. Carcinoma cases were subdivided into tubular and solid components on the basis of tumour growth patterns. Immunopositivity for Bcl-2, ER, and PR in tubular components was significantly higher than in the solid category, being negatively associated with histological grading. Immunoreactivity scores revealed that Bcl-2 in the tubular group was positively correlated with PR but not ER, while its expression in normal and hyperplastic endometrium was closely linked with both. Apoptotic and mitotic indices (AI and MI) were both significantly lower in tubular than in solid areas. In the tubular areas, AI values were significantly lower in the subgroup with a high level of Bcl-2 expression than in either low-level or negative groups. These results indicate that Bcl-2 expression may play a central role in the inhibition of apoptosis in endometrial carcinoma, in particular those cases with tubular components, possibly being associated with PR rather than ER. Changes in the propensity for apoptosis may be related to alterations of tumour growth pattern and of features of differentiation.

KEY WORDS—**Bcl-2; ER; PR; endometrial carcinoma; apoptosis**

INTRODUCTION

Physiological events in the normal menstrual cycle are closely regulated by sex steroid hormones. Alteration of the normal balance, with stimulation of the endometrium by oestrogen without the differentiating effect of progesterone, is widely accepted as a primary aetiological factor associated with the development of endometrial hyperplasia and carcinoma.[1] Some studies have documented that hormone receptor status [oestrogen (ER), progesterone (PR)] is linked with histological differentiation and response to therapy, a determining factor in the prognosis of endometrial carcinomas.[2–4]

Expression of the bcl-2 gene has been demonstrated in normal endometrium during the menstrual cycle, being possibly associated with oestrogen and progesterone status.[5] Recently, Chan *et al.*[6] showed that Bcl-2 expression decreased in the sequence leading from hyperplastic endometrium, through atypical hyperplasia to poorly differentiated carcinoma, suggesting a role for Bcl-2 in the natural history of endometrial neoplasms. However, the relationship between Bcl-2 expression and hormone receptor status in endometrial carcinoma is still unclear.

Apoptotic bodies are frequently observed in a variety of human malignant tumours, positively correlating with tumour differentiation and progression.[7,8] While Bcl-2 is a known inhibitor of apoptosis, thus prolonging cellular life-span, its aberrant expression may thereby allow the accumulation of additional genetic alterations, potentially contributing to tumour development.[9]

In the present study, to clarify the possible role of Bcl-2 in tumour tissue kinetics, we investigated normal, hyperplastic, and malignant endometrium, using immunohistochemical procedures to demonstrate Bcl-2, ER, and PR, and compared the results with findings for apoptosis and cell proliferation.

MATERIALS AND METHODS

Case material

A total of 103 cases of endometrial carcinoma (endometrioid type), surgically resected at the Kitasato University Hospital from 1988 to 1995, were investigated, along with 26 cases of simple or complex hyperplasia without atypia and 15 samples of normal endometrium in the atrophic or proliferative phases. All tissues were routinely fixed in 10 per cent formalin and embedded in paraffin wax. Histological diagnosis was performed according to the criteria of the International Federation of Gynecology and Obstetrics (1988).[10] Since this histopathological classification is based on the proportion of non-squamous or non-morular solid growth patterns, the carcinoma cases were further subdivided into two groups, tubular and solid, in accordance with their growth patterns. Carcinoma cases comprised 59 G1 lesions (all tubular components only), 19 G2 (all featuring both components), and 25 G3 (10 with both components and 15 with only solid components).

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CCC 0022–3417/96/110275–08

Received 29 January 1996
Accepted 7 May 1996

Immunohistochemistry

Immunohistochemistry was performed using a combination of the microwave-oven heating and the standard streptavidin–biotin–peroxidase complex [Histofine SAB-PO (M) kit, Nichirei Co., Tokyo, Japan] methods on 4 μm thick sections. Briefly, after deparaffinization, the slides were heated in 10 mM citrate buffer (pH 6·0) for three 5-min cycles using a microwave oven and then incubated overnight at 4°C with optimum dilution of primary antibodies. The antibodies used were anti-human bcl-2 mouse monoclonal antibody (× 100 dilution, Dako, Copenhagen, Denmark), anti-oestrogen receptor mouse monoclonal antibody (× 60 dilution, Novocastra Lab. Ltd., Newcastle, U.K.), and anti-progesterone receptor mouse monoclonal antibody (× 60 dilution, Novocastra Lab. Ltd., Newcastle, U.K.). To confirm the immunospecificity of each antibody, 0·01 M phosphate-buffered saline or normal mouse serum (× 500 dilution) was supplied instead of primary antibodies as a negative control.

Scoring for Bcl-2, ER, and PR immunoreactivity

Scoring of the immunohistochemistry results was performed according to the methods described by Sinicrope *et al.*[11] with minor modifications. Based on the percentages of Bcl-2, ER, and PR immunopositive cells in the total number of tumour cells, subdivision was into five categories as follows: 0, all negative; 1, <10 per cent positive cells; 2, 10–30 per cent; 3, 30–50 per cent; and 4, >50 per cent. The immunointensity was also subclassified into four groups in comparison with internal controls, for example, infiltrating lymphocytes as internal positive controls for Bcl-2 and normal endometrial epithelium or stromal cells for ER or PR, as follows: 0, negative; 1+, week; 2+, moderate; and 3+, strong. The internal positive controls were set as 3+. Immunoreactive scores for each case were produced by multiplication of the values for the two parameters.

Apoptotic and mitotic indices

Applying the distinctive morphological features of apoptotic cells, with marked condensation of chromatin and cytoplasm with or without nuclear fragments, as described by Kerr *et al.*,[12] their incidence was examined in haematoxylin and eosin (H & E)-stained sections under high-power (× 40 objective and × 10 ocular) magnification. Apoptotic index (AI) values were calculated after examining at least 2500 nuclei in five randomly selected fields from both tubular and solid components for each case. Areas of severe inflammatory cell infiltration and necrosis were excluded, since some doubtful cells were observed in such regions. Mitotic index (MI) values were calculated in a similar manner, counting mitotic figures.

Statistics

Statistical analysis of data for AI, MI, and immunoreactive scores of Bcl-2, ER, and PR was performed using the Mann–Whitney *U*-test. The association between Bcl-2 and ER or PR was analysed using the Pearson's correlation coefficient. In addition, the incidence of immunopositivity for Bcl-2, ER, and PR was examined by the chi-square test. The cut-off for statistical significance was defined as $P<0·05$.

RESULTS

Bcl-2 expression

In all 15 normal endometrial samples, intense Bcl-2 immunoreactivity (3+) with uniform cytoplasmic and perinuclear staining was found in glandular cells (Fig. 1A), the basal layer reacting more strongly than the functional zone. Stromal cells in the basal layer were weakly positive (1+), while those in the functional zone lacked Bcl-2 immunoreactivity. The smooth-muscle cells in the myometrium strongly bound Bcl-2 antibodies (3+), while the arterioles showed weak immunoreactivity (1+) or were negative.

High-level and uniform Bcl-2 immunoreactivity (3+) in hyperplastic endometrial glandular cells without atypia was detected in 25 of 26 (96·2 per cent) cases (Fig. 1B). Bcl-2 positivity in the other components, including the stromal cells, arterioles, and myometrium, resembled that in the normal case.

Variation in Bcl-2 immunointensity (0 to 3+) and a heterogeneous distribution of positive cells were observed in carcinomas (Figs 1C and 1D). Immunoreactivity was found for 69 or 88 (78·4 per cent) tubular and 22 of 44 (50 per cent) solid components, the difference being significant ($P=0·0009$). Tumour elements showing squamous differentiation within tubular components lacked Bcl-2 immunoreactivity.

ER and PR expression

In the 15 normal endometrial samples and 26 endometrial hyperplasias, ER and PR immunoreactivity (1+ to 3+) was detected in glandular, stromal, and myometrial cells, with distinct nuclear staining, although some glandular cells were negative for one or both of the receptors. Arterioles were mostly negative.

Heterogeneous staining (0 to 3+) and location of ER- and/or PR-positive cells were noted in carcinoma cases, with the immunoreactivity of PR predominating over that of ER. ER immunoreactivity was found for 49 of 88 (55·7 per cent) tubular and 7 of 44 (15·9 per cent) solid components, the difference being significant ($P=0·0001$). The rate of PR positivity, 76 of 88 (86·4 per cent) tubular components, was also significantly greater than the 23 of 44 (52·3 per cent) for solid growth pattern cases ($P<0·0001$). Areas with squamous differentiation in carcinomas were negative for both ER and PR antibodies, as well as for Bcl-2 immunoreactivity.

A strong correlation between ER and PR immunoreactive scores was found for hyperplastic lesions, while a loss of this correlation was observed in carcinomas, in line with malignant grading (Table I).

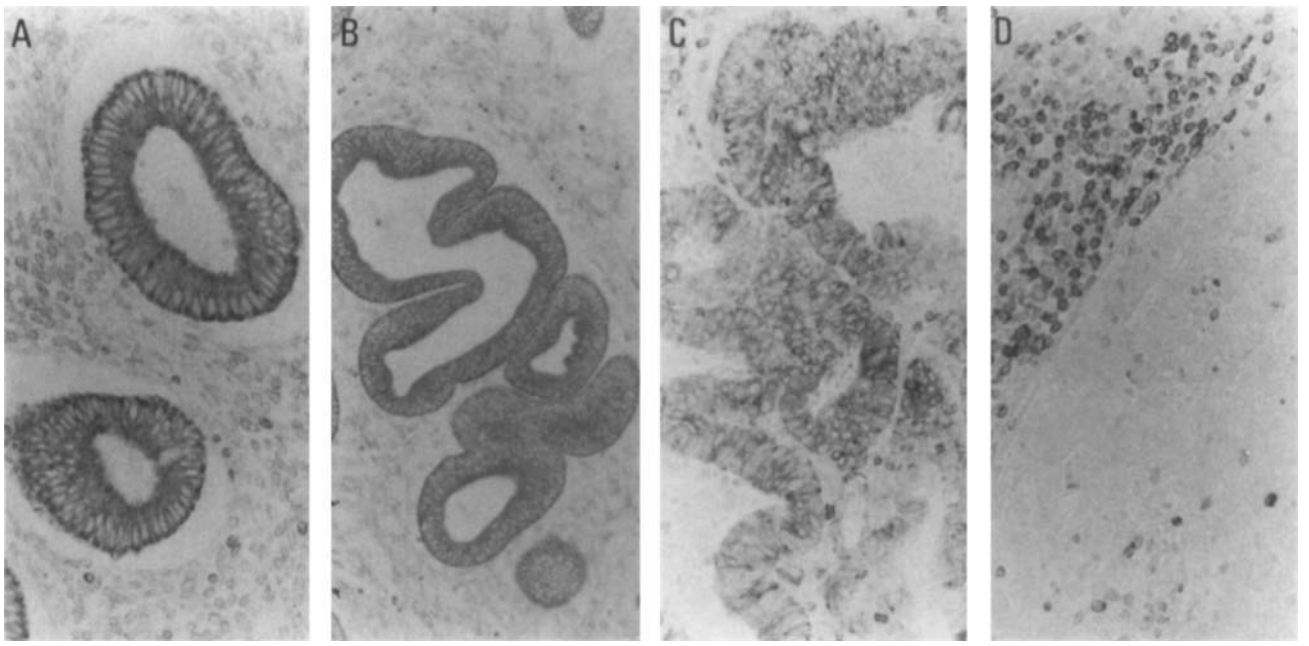

Fig. 1—Immunohistochemistry of Bcl-2. (A) Note the strong immunoreactivity in normal endometrial glandular epithelium and a weak reaction in stromal cells. × 400. (B) Hyperplastic endometrial glands also show strong immunoreactivity. × 200. (C) G1 endometrial carcinoma. Heterogeneous immunoreactivity is observed in tubular components. × 400. (D) G3 endometrial carcinoma. Solid component (lower right) clearly lacks Bcl-2 immunoreactivity, while strongly positive staining is observed in lymphocytes (upper left). × 400

Table I—Correlations between Bcl-2, ER, and PR immunoreactivity

			Bcl-2 vs. ER		Bcl-2 vs. PR		ER vs. PR	
		n	Pearson's correlation coefficient (*r*)	*P*	Pearson's correlation coefficient (*r*)	*P*	Pearson's correlation coefficient (*r*)	*P*
Em Ca	G1	59	0·135	0·307	0·567	<0·0001	0·341	0·008
(tubular	G2	19	0·008	0·973	0·476	0·039	0·31	0·196
component)	G3	10	0·056	0·878	0·232	0·519	0·253	0·482
Total		88	0·192	0·073	0·516	<0·0001	0·373	0·0003
Em Ca	G2	19	0·452	0·052	0·663	0·002	0·529	0·02
(solid component)	G3	25	0·07	0·74	0·171	0·413	0·23	0·267
Total		44	0·234	0·126	0·248	0·105	0·387	0·009
Hyperplasia		26	0·425	0·03	0·581	0·001	0·682	0·0001
Normal		15	0·495	0·06	0·595	0·019	0·262	0·345

Em Ca=endometrial carcinoma; ER=oestrogen receptor; PR=progesterone receptor; *n*=number of cases.

Correlations between Bcl-2 and ER or PR expression

Areas of Bcl-2-positive cells corresponded to those demonstrating ER and PR immunolabelling in normal and hyperplastic endometrium (Table I). In contrast, the distribution of Bcl-2-positive cells in carcinomas generally overlapped PR immunoreactivity, better than ER staining (Fig. 2). Thus, Bcl-2 was positively correlated with PR in tubular although not in solid components, while no association with ER positivity was noted (Table I).

Immunoreactivity scores for Bcl-2, ER, and PR were all related to either histological growth pattern or grading of the carcinomas (Fig. 3).

Apoptotic and mitotic indices

Apoptotic cells demonstrated markedly condensed nuclei and homogeneous eosinophilic cytoplasm, with or without nuclear fragments (apoptotic bodies) and were generally separated from the surrounding cells by a clear halo. Clusters of condensed nuclear fragments were also observed in tumour nests (Fig. 4). Careful observation was necessary, since some lymphocytes infiltrating endometrial epithelium exhibited a similar appearance to apoptotic cells, due to the clear chromatin patterns in nuclei.

The AIs of normal and hyperplastic epithelium were $0{\cdot}14 \pm 0{\cdot}21$ per cent (mean ± SD) and $0{\cdot}43 \pm 0{\cdot}27$ per

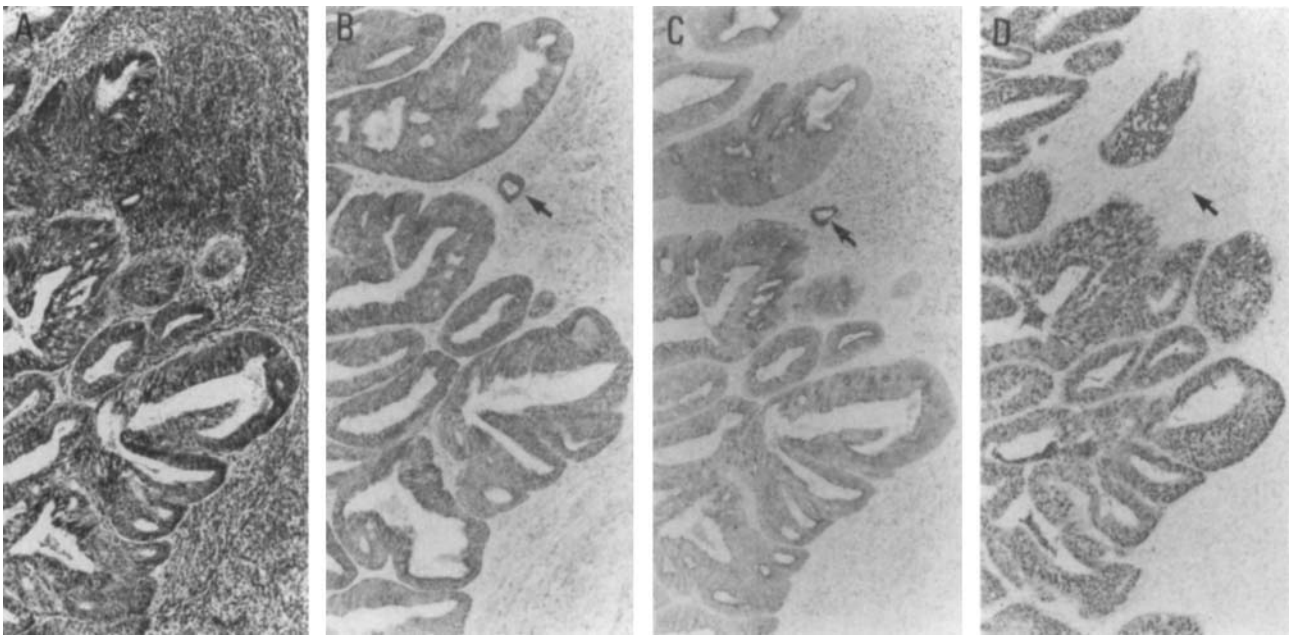

Fig. 2—Serial sections through an endometrial carcinoma. (A) Tubular elements are shown with invasion into the myometrium. H & E, × 100. (B) Note the strong immunoreactivity for Bcl-2 in both the tumour and the normal endometrial glandular cells (indicated by an arrow). × 100. (C) ER immunoreactivity is weak or absent in tumour cell nuclei but strong in normal glandular cells (indicated by an arrow). × 100. (D) Strong immunoreactivity for PR in the tubular components of the tumour but not in normal glandular cells (indicated by an arrow). × 100

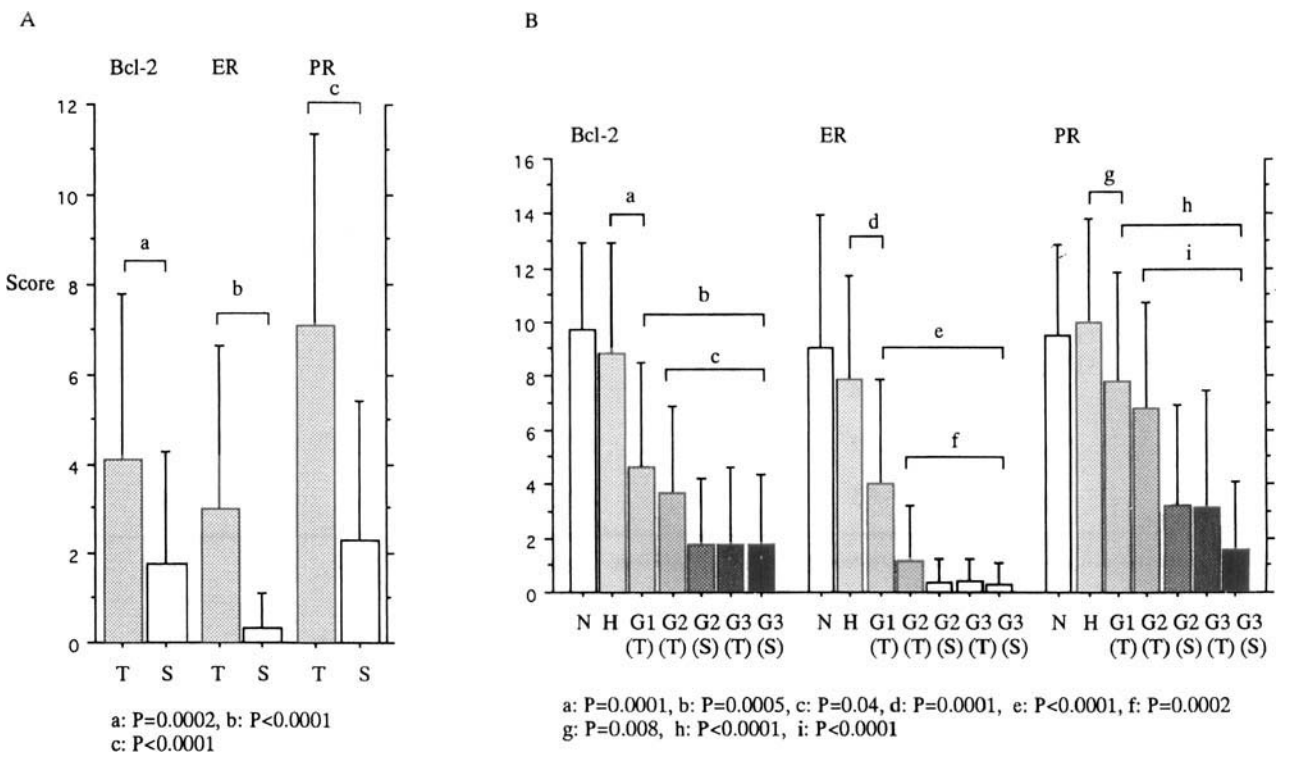

Fig. 3—Immunoreactivity scores for Bcl-2, ER, and PR in normal (N) and hyperplastic (H) endometrium and in carcinomas (T, tubular components; S, solid components; G1, grade 1; G2, grade 2; G3, grade 3). The data are mean values ± SD

cent, respectively. In carcinomas, the average value for tubular components was 0·89 ± 0·52 per cent (G1, 0·83 ± 0·53 per cent; G2, 1·02 ± 0·46 per cent; G3, 1·04 ± 0·48 per cent) and for the solid category 1·29 ± 0·73 per cent (G2, 1·14 ± 0·58 per cent; G3, 1·40 ± 0·85 per cent).

The MIs of normal and hyperplastic lesions were 0·03 ± 0·06 and 0·10 ± 0·09 per cent, respectively, rising

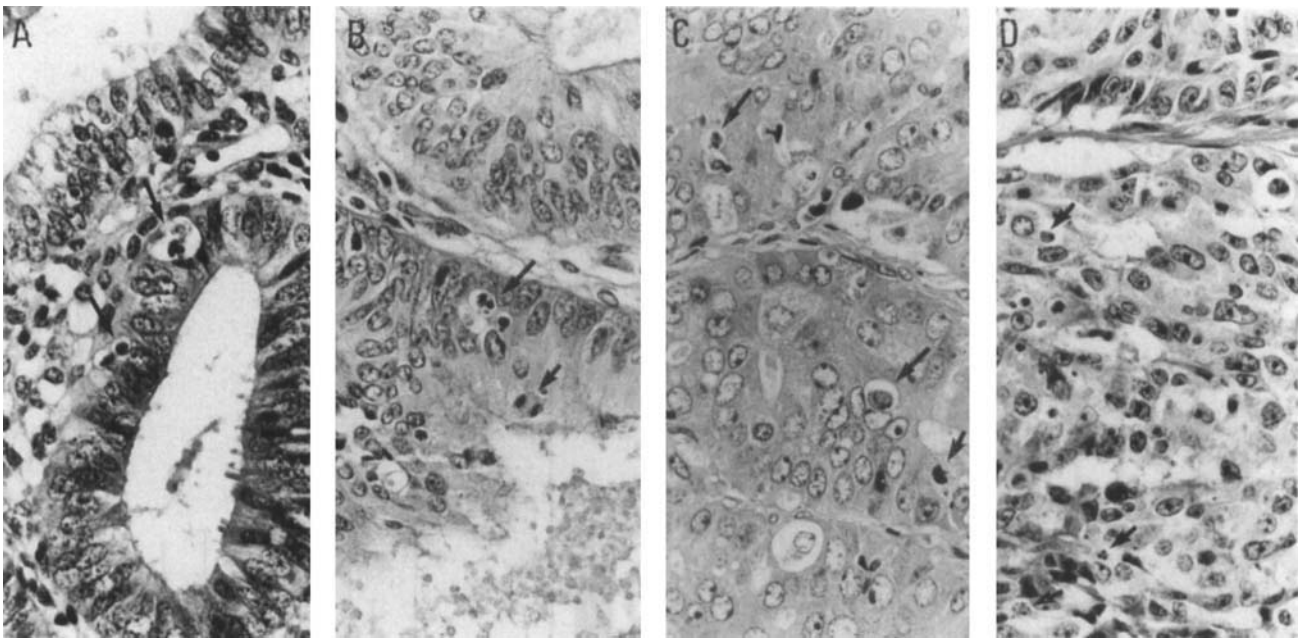

Fig. 4—Apoptotic and mitotic cells. (A) Apoptotic cells (indicated by arrows) demonstrate markedly condensed nuclei and cytoplasm and separation from the surrounding cells by haloes in hyperplastic epithelium. × 640. (B, C) Apoptotic (indicated by long arrows) and mitotic cells (indicated by short arrows) observed in G1 (B) and G2 (C) carcinomas. × 640. (D) Many apoptotic cells (indicated by arrows) are evident in a G3 carcinoma. × 640

to an average of 0·38 ± 0·29 per cent (G1, 0·33 ± 0·27 per cent; G2, 0·48 ± 0·32 per cent; G3, 0·53 ± 0·33 per cent) for tubular components of carcinomas and 0·69 ± 0·41 per cent (G2, 0·48 ± 0·25; G3, 0·84 ± 0·45 per cent) for solid areas.

In carcinomas, the increase of AI in line with malignant grading was more pronounced than that for MI, with an inverse correlation with AI/MI being noted (Fig. 5). However, a positive correlation between AI and MI was noted for both tubular (r=0·43, P<0·0001) and solid (r=0·58, P<0·0001) components (Figs 6A and 6B). Both the AI and the MI in tubular lesions were significantly lower than in solid areas, while the AI/MI in the former was statistically greater than in the latter (Fig. 6C).

Correlation between the AI or MI and immunoreactivity for Bcl-2, ER, and PR

Significant differences in AIs were found between areas of high-level Bcl-2 expression (score ≧7) and either low-level (score ≦6) or negative regions in the tubular components of carcinomas, but not for the solid category (Fig. 6D). No statistically significant relationship was observed between Bcl-2 and MI (data not shown) and neither ER nor PR immunoreactivity scores were significantly associated with AI or MI.

DISCUSSION

In the present study, classification of endometrial carcinomas (endometrioid type) not only into the three conventional categories (G1, G2, and G3), but also into two subgroups (tubular and solid components) allowed clear differentiation in terms of the immunohistochemical parameters investigated. The proportions of both tubular and non-squamous or non-morular morphological solid growth patterns are considered to be of clinicopathological prognostic significance,[13] as well as being closely linked to the ER and/or PR status.[2] Our results indicate significantly higher proliferative activity and lower immunopositivity for ER and PR in solid than in tubular components, implying a higher potential for aggressive behaviour and malignancy.

A relationship between Bcl-2 and steroid hormone regulation has been widely documented, expression of the proto-oncogene being significantly correlated with both ER and PR immunoreactivity in breast carcinomas,[14] and inversely correlated with androgen in prostate carcinomas.[15] Otsuki *et al.*[16] demonstrated that the cyclic Bcl-2 expression pattern in endometrial glandular cells is related to those for ER and PR in the normal menstrual cycle, suggesting regulation of Bcl-2 expression by ovarian hormones and especially oestrogen, in line with the fact that synthesis of PR is induced by oestrogen action in hormonally responsive cells.[17]

In the present study, Bcl-2 expression was closely correlated with ER and PR immunoreactivity in normal and hyperplastic endometrial glandular cells, supporting an oestrogen-dependent regulation.[5,16] However, in carcinomas, a significantly positive relationship was noted only between Bcl-2 and PR immunoreactivity and was limited to the tubular components, despite the existence of a general correlation between ER and PR immunoreactivity. We can thus speculate that the regulation of Bcl-2 within endometrial carcinomas is more complex than in normal or hyperplastic epithelium, perhaps being modulated by multiple regulatory proteins. In addition, the finding that Bcl-2 expression disappeared

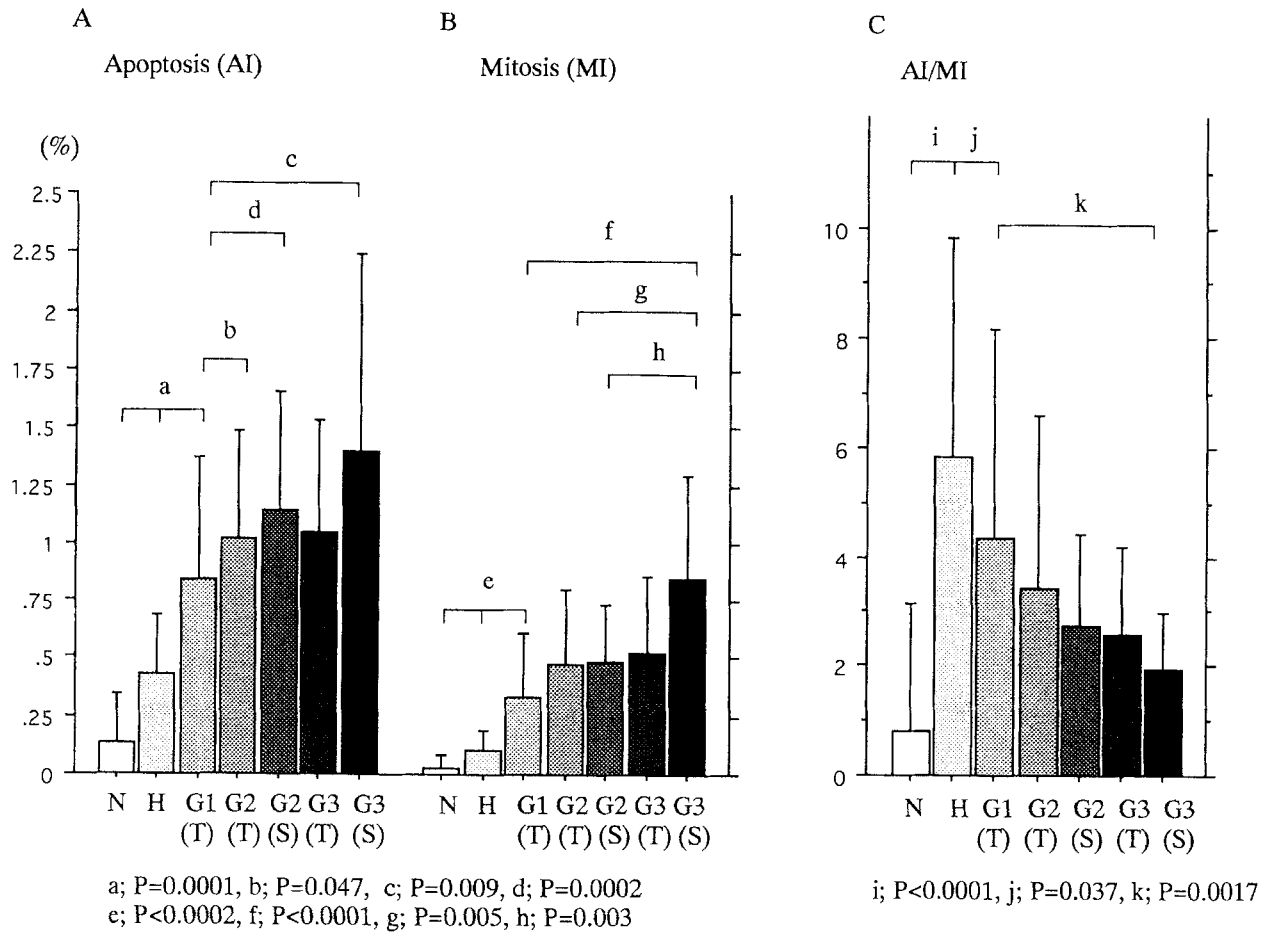

Fig. 5—AI, MI and AI/MI ratios for normal (N) and hyperplastic (H) endometrium and carcinomas (T, tubular; S, solid; G1, grade 1; G2, grade 2; G3, grade 3). The data are mean values ± SD

during squamous differentiation within tubular growth carcinomas is of interest, since down-regulation of Bcl-2 appears to be part of the differentiation pathway in the HL-60 promyelocytic cell line.[18]

Two distinctly different pathways of endometrial carcinogenesis have been considered.[19] One is related to hyperoestrogenism, with development from hyperplastic endometrium (i.e., hyperplasia–carcinoma sequence). The other appears to be independent of direct hormonal effects, with development from normal (or atrophic) endometrium, the affected patients showing a poor prognosis in comparison with those having hormone-dependent carcinomas. Strong Bcl-2 immunoreactivity in hyperplastic glandular cells suggests that Bcl-2 may play an important role in a relatively early stage of the endometrial hyperplasia–carcinoma sequence. Tomei *et al.*[20] have asserted that the inhibition of apoptosis may be directly linked to the mechanism of tumour promotion.

In this study, the presence of ER and PR was investigated by immunohistochemical methods alone, since an apparent correlation between standard biochemical and immunohistochemical techniques for detection of these receptors has been demonstrated.[21] Creasman recently proposed that PR status may have greater significance for predicting prognosis than ER status alone, and may be a better predictor of survival than other well-recognized prognostic factors, such as cervical involvement, linear age, and extrauterine metastasis.[22] Considering that Bcl-2 expression is a favourable factor in breast and non-small cell lung carcinomas,[23–25] it is very possible that it may also be related to the biological behaviour of endometrial carcinomas, in association with altered hormone receptor action. Further studies in this area are clearly warranted.

Cells with a high proliferative activity commonly appear likely to undergo apoptosis,[26,27] while withdrawal of trophic hormonal stimulation also leads to apoptosis.[28] The report of a positive correlation between its frequency and the histological grade of prostatic carcinomas indicated that it may be a feature of increased malignant potential.[29] In our present study, AIs were significantly increased through normal to malignant endometrium and apparently correlated with histological grade in carcinomas, suggesting that a change in the propensity for apoptosis may be related to the development or progression of endometrial carcinoma and loss of differentiated features. Moreover, since the AI/MI ratio reflects the balance of cell deletion and proliferation, its reduction with alteration from tubular to solid patterns is another pointer to a shift in tissue kinetics towards aggressive growth.

Bcl-2 is expressed in long-lived cells and leads to cell accumulation in the G0 phase of the cell cycle by

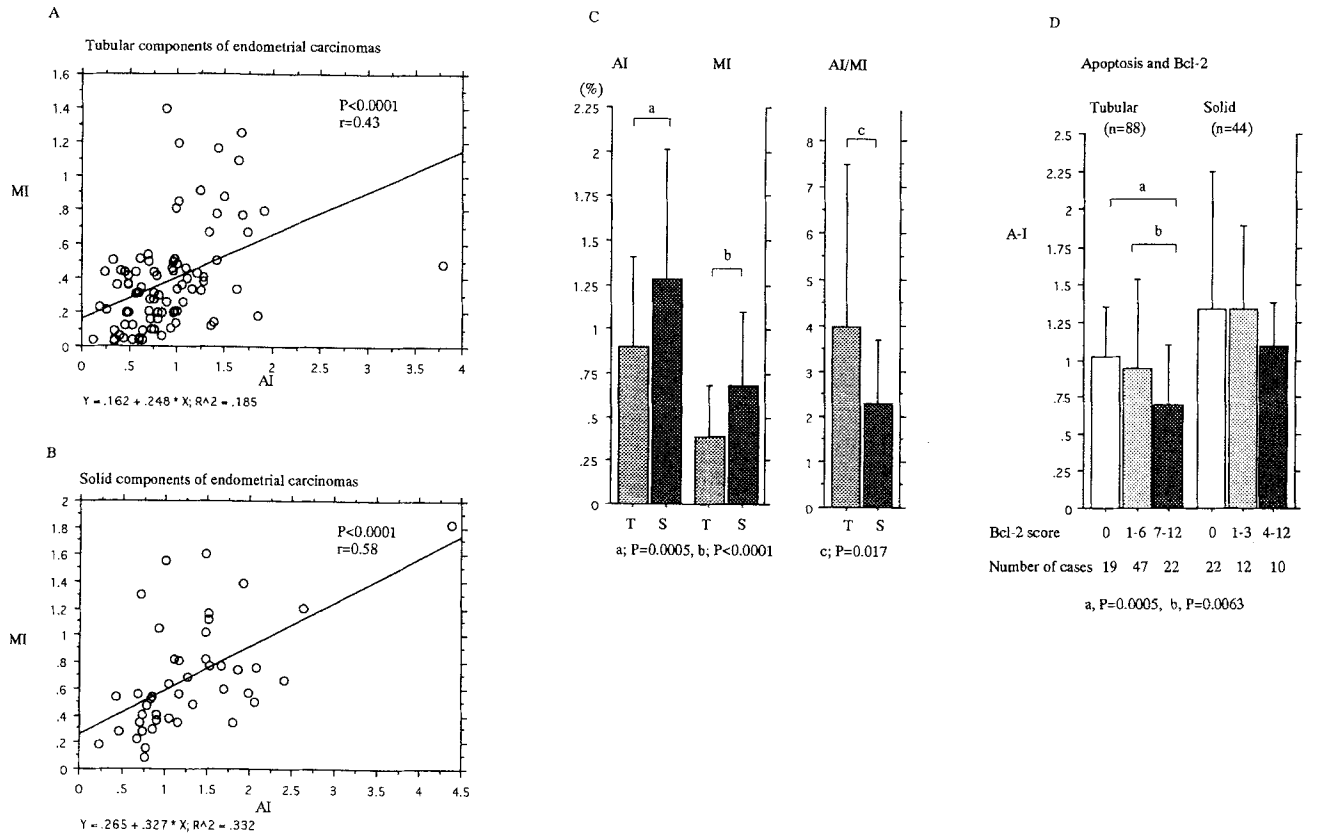

Fig. 6—(A, B) Correlations between apoptotic (AI) and mitotic indices (MI) in tubular (T) and solid (S) components of endometrial carcinomas. (C) Data for AI, MI, and AI/MI (mean values ± SD). (D) Relationship between Bcl-2 immunoreactivity scores and apoptotic indices in carcinomas (T, tubular; S, solid). The data are mean values ± SD

inhibition of apoptosis.[30,31] Colorectal carcinomas with a high percentage of cells expressing Bcl-2 are significantly more likely to have low AIs than those with little or no Bcl-2.[11] Breast carcinomas exhibiting malignant histological features with rapid cell proliferation are usually Bcl-2-negative.[23] Our finding that high-level Bcl-2 expression in tubular components is inversely correlated with AIs but not MIs, despite the positive relationship between the latter, supports the hypothesis that Bcl-2 is responsible for inhibition of apoptosis. The lack of any association in solid components may be simply a reflection of a more malignant character.

In conclusion, a high level of Bcl-2 expression may play a central role in the inhibition of apoptosis in endometrial carcinomas, in particular in tubular components, being more closely associated with PR than ER status. In addition, changes in the propensity for apoptosis may be directly related to alteration of tumour growth pattern and to features of differentiation.

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