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DOWN-REGULATION OF bcl-2 EXPRESSION IS CLOSELY RELATED TO SQUAMOUS DIFFERENTIATION AND PROGESTERONE THERAPY IN ENDOMETRIAL CARCINOMAS

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SUMMARY

To clarify whether changes in bcl-2 protein (bcl-2) expression are directly linked to differentiation, an immunohistochemical investigation was carried out on areas of squamous differentiation within 38 endometrial carcinomas (25 grade 1 and 13 grade 2 cases) as well as eight grade 1 carcinomas after progesterone therapy. Such areas of altered differentiation were subdivided into foci of squamous metaplasia (Sq-M) and morules. Expression of oestrogen and progesterone receptors (ER and PR) and cell proliferation were also examined. Immunoreactivity scores for bcl-2 in Sq-M foci were significantly lower than in the surrounding tumour regions, in line with reduced staining for ER and PR and Ki-67 labelling indices (LIs), while morule values were intermediate. After progesterone therapy, a marked decrease in bcl-2 immunoreactivity was found in the response group, along with low Ki-67 LIs and tumour cell maturation. These results indicate that in endometrial carcinoma, down-regulation of bcl-2 expression may be closely linked with squamous differentiation, in line with changes in ER and PR expression, as well as in cell proliferation. In addition, bcl-2 expression appears to be down-regulated by progesterone therapy through tumour cell maturation, indicating that bcl-2 may be a clinically useful marker of hormone therapy effects. © **1997 by John Wiley & Sons, Ltd.**

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KEY WORDS—**endometrial carcinoma; bcl-2; ER; PR; Ki-67; hormone therapy**

INTRODUCTION

The bcl-2 protein (bcl-2) plays an important role in cell regulation, functioning to inhibit apoptosis (programmed cell death);[1,2] a close association between its overexpression and tumourigenesis has been proposed in a variety of human tissues.[3,4] This is based on the reasoning that prolonging life span increases the risk of secondary genetic alteration, resulting in malignant transformation.[5] We have recently demonstrated that bcl-2 expression in endometrial carcinomas is associated with a low apoptotic index and also with progesterone receptor immunoreactivity, supporting a possible role in tumour development and progression.[6]

It is generally accepted that bcl-2 is closely linked with morphogenesis and differentiation. Novack and Korsmeyer[7] demonstrated that during embryogenesis, bcl-2 expression becomes increasingly restricted to certain cell types within each tissue as organ differentiation proceeds, being found, for example, throughout the undifferentiated intestinal epithelium, but only in the progenitor zones as cells mature. Lu *et al*.[8] also asserted that bcl-2 expression is down-regulated once undifferentiated stem cells have entered into the process of terminal differentiation.

Elton[9] earlier noted definite evidence of secretory activity and a high incidence of squamous metaplasia in well-differentiated endometrial carcinomas appearing after the menopause, suggesting that this tumour response is due to a progesterone-like substance. Hormone therapy for endometrial carcinoma was subsequently introduced by Kelley and Baker[10] on the basis of profound maturation stimulating effects of progesterone.

In this study of bcl-2 expression and differentiation in endometrial carcinomas, we have investigated squamous differentiation within tumours and the histological changes induced in tumour cells by hormone therapy. In addition, the expression of oestrogen and progesterone receptors (ER and PR) and proliferative activity were examined.

MATERIALS AND METHODS

Case selection

Histological preparations were reviewed from 121 hysterectomy specimens of endometrial carcinoma in the case records of Kitasato University Hospital. The lesions were diagnosed in the period from 1990 to 1995, according to the histological criteria of the International Federation of Gynecology and Obstetrics[11] and the

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World Health Organization histological classification.[12] Squamous differentiation within tumours was subdivided into two categories, squamous metaplasia (Sq-M) and morule foci. Briefly, Sq-M foci were defined as follows: existence of keratinization; existence of intercellular bridging or clear cell–cell borders; abundant eosinophilic cytoplasm; and reduced size of nuclei (N) in relation to the cytoplasm (C) (N/C ratio). Morules were designated as areas consisting of spindle-shaped cells forming growth sheets with unclear cell–cell borders. The cases with squamous differentiation investigated comprised 25 (36·2 per cent) or 61 grade (G) 1 and 13 (41·9 per cent) G2 carcinomas but none of 21 G3 carcinomas. All tissues were routinely fixed in 10 per cent formalin and embedded in paraffin.

Twelve G1 endometrial carcinomas without extrauterine extension in patients receiving hormone therapy before hysterectomy were also selected from the patients' charts of Kitasato University Hospital for the period 1988–1996. Biopsy samples containing enough carcinoma elements taken on at least two occasions before medroxyprogesterone acetate (MPA) treatment and at least four after therapy were available in eight cases, the other four being excluded because it was impossible to examine sequential morphological changes resulting from the hormone therapy. Of the eight cases investigated, four finally underwent hysterectomy and the other four were continued on MPA therapy.

All sections of biopsy specimens stained by haematoxylin and eosin (H & E) were reviewed and morphological changes during hormone therapy were evaluated in terms of the following four parameters: cellularity; nuclear rearrangement (mono- or multiple integrated layers); increase of eosinophilic change in the cytoplasm; and N/C ratio. Therapeutic efficacy (TE) was assessed by counting the numbers of altered parameters. For example, when reduced cellularity and a decreased N/C ratio were found in more than 50 per cent of the tumour cells in biopsy specimens after hormone therapy, in comparison with samples before treatment, the grade was defined as 2.

Immunohistochemistry

Immunohistochemistry was performed using a combination of microwave-oven heating and the standard streptavidin–biotin–peroxidase complex (LSAB kit, Dako, Copenhagen, Denmark) method. Briefly, slices 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 dilutions 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 (× 80 dilution; Novocastra Lab. Ltd., Newcastle, U.K.), anti-progesterone receptor mouse monoclonal antibody (× 80 dilution; Novocastra Lab. Ltd., Newcastle, U.K.), and rabbit anti-human Ki-67 antigen (× 150 dilution; Dako, Copenhagen, Denmark). To confirm the immunospecificity of each antibody, 0·01 M phosphate-buffered saline or normal mouse serum (× 500 dilution) for bcl-2, ER, and PR immunohistochemistry, and normal rabbit serum (× 200 dilution) for Ki-67 assay were supplied instead of primary antibodies as negative controls.

Scoring for bcl-2, ER, and PR immunoreactivity

Scoring of immunohistochemistry results was performed according to the method previously described.[6] Briefly, the percentage of immunopositive cells in the total tumour cell population was subdivided 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 intensity of immunostaining was also subclassified into four groups in comparison with internal controls, using infiltrating lymphocytes for bcl-2 and normal endometrial glands and myometrium for ER and PR, as follows: 0, negative; 1+, weak; 2+, moderate; and 3+, strong. The internal controls were set as 3+. Immunoreactivity scores for each case were produced by multiplication of two values.

Ki-67 labelling index (LI)

To determine Ki-67 LIs of carcinoma areas, slides were randomly moved and five fields were selected for each case using a × 40 objective and a × 10 ocular lens. Ki-67-positive nuclei were counted for at least 2000 tumour cells in total and LIs calculated as number per 100 cells. For Ki-67 LIs of areas of squamous differentiation, these lesions were selected and LI values were calculated after examining nuclei in all areas of squamous differentiation in each case (average values, 952 nuclei; range 310–1899).

Suppression rate (SR)

Percentage changes in immunoreactivity scores induced by hormone therapy were defined as suppression rates (% bcl-2, % ER, % PR, and % Ki-67) calculated in each case as follows: [1 − (average value for the immunoreactivity score or LI in several biopsy specimens after MPA therapy, divided by that before the treatment)].

Statistics

Statistical comparison of data for Ki-67 LIs and immunoreactivity scores for bcl-2, ER, and PR were performed using the Mann–Whitney *U*-test. The association between SR and TE was analysed using the Pearson correlation coefficient. The cut-off for statistical significance was defined as $P<0·05$.

RESULTS

Histological findings for areas of squamous differentiation within endometrial carcinomas

The majority of Sq-M foci, resembling terminally differentiated keratinocytes, were detected in tubular components of carcinomas, showing sharp demarcation

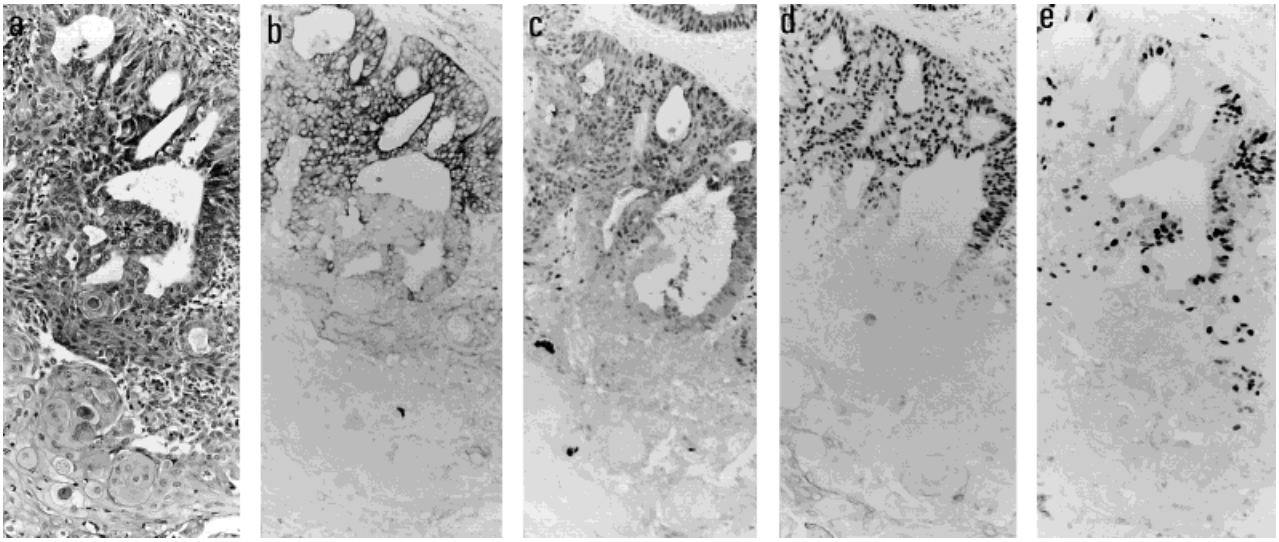

Fig. 1—Serial sections through a Sq-M focus within an endometrial carcinoma. (a) Note the change from a grade 1 carcinoma (upper half) to a Sq-M focus (lower half), with a relatively sharp demarcation. H & E staining. Immunohistochemistry for bcl-2 (b), ER (c), PR (d), and Ki-67 (e). Note the strong immunoreactivity of each antibody in the tubular component but not in the Sq-M focus

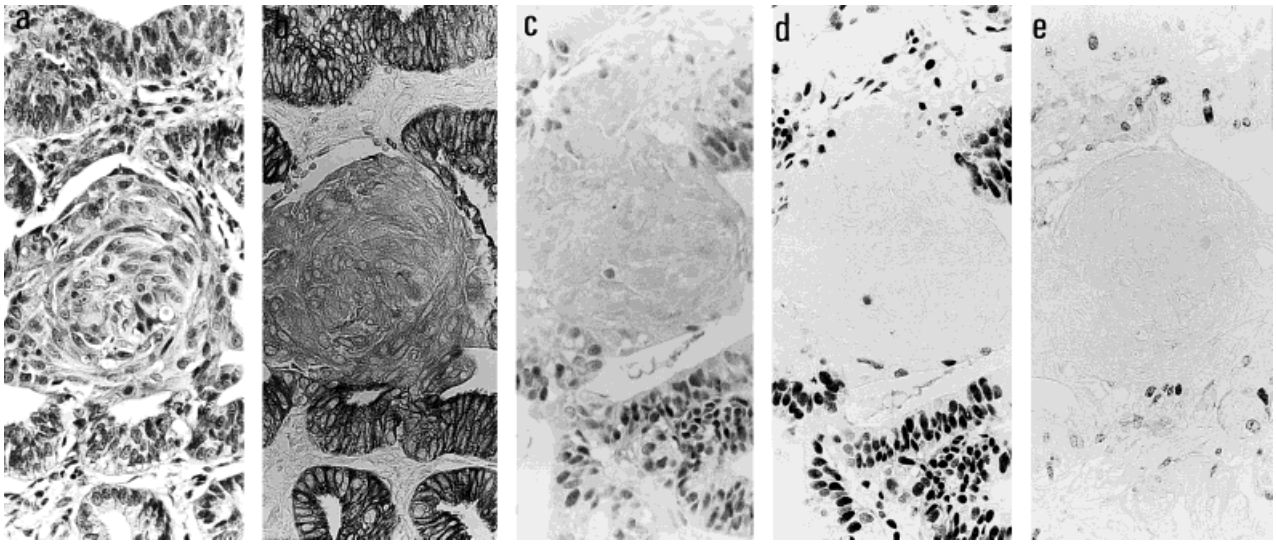

Fig. 2—Serial sections through a morule within an endometrial carcinoma. (a) The morule consists of spindle-shaped cells with unclear cell–cell borders. H & E staining. (b) bcl-2 immunoreactivity in the morule is relatively weak in comparison with that in the primary tumour. Immunoreactivity for ER (c), PR (d), and Ki-67 (e) is completely negative, in contrast to the positive immunostaining for each in the tubular components

from the surrounding cancer cells (Fig. 1a). Rarely, tumour cells in solid growth areas demonstrated an abrupt transformation to Sq-M foci. Morules were also predominantly found in tubular elements, sometimes accompanied by central necrosis (Fig. 2a). Multiple areas of squamous differentiation were frequently found in one tumour.

Of 38 positive carcinomas, Sq-M foci were found in 26 cases, morules in seven cases, and both in five cases.

Comparison of bcl-2, ER and PR expression and Ki-67 LI between primary tumours and areas of squamous differentiation

bcl-2 immunoreactivity was cytoplasmic (Figs 1b and 2b), in contrast to distinct nuclear immunostaining of ER and PR (Figs 1c, 1d, 2c, and 2d). Strong immunoreactivity for bcl-2, ER, and PR in carcinomas frequently became abruptly negative in the Sq-M foci and morules, with the exception of bcl-2 in morule lesions (Figs 1 and 2).

Immunoreactivity scores of ER and PR were clearly decreased in areas of squamous differentiation compared with surrounding carcinoma, the difference being significant in both cases ($P<0{\cdot}0001$). The bcl-2 scores for Sq-M foci were also significantly decreased ($P<0{\cdot}0001$), while morule values were intermediate (Fig. 3).

Data for differences in immunopositivity for bcl-2, ER, and PR between primary tumours and areas of squamous differentiation are summarized in Table I. In most cases, immunoreactivity was clearly decreased in both Sq-M foci and morules, with the exception of bcl-2

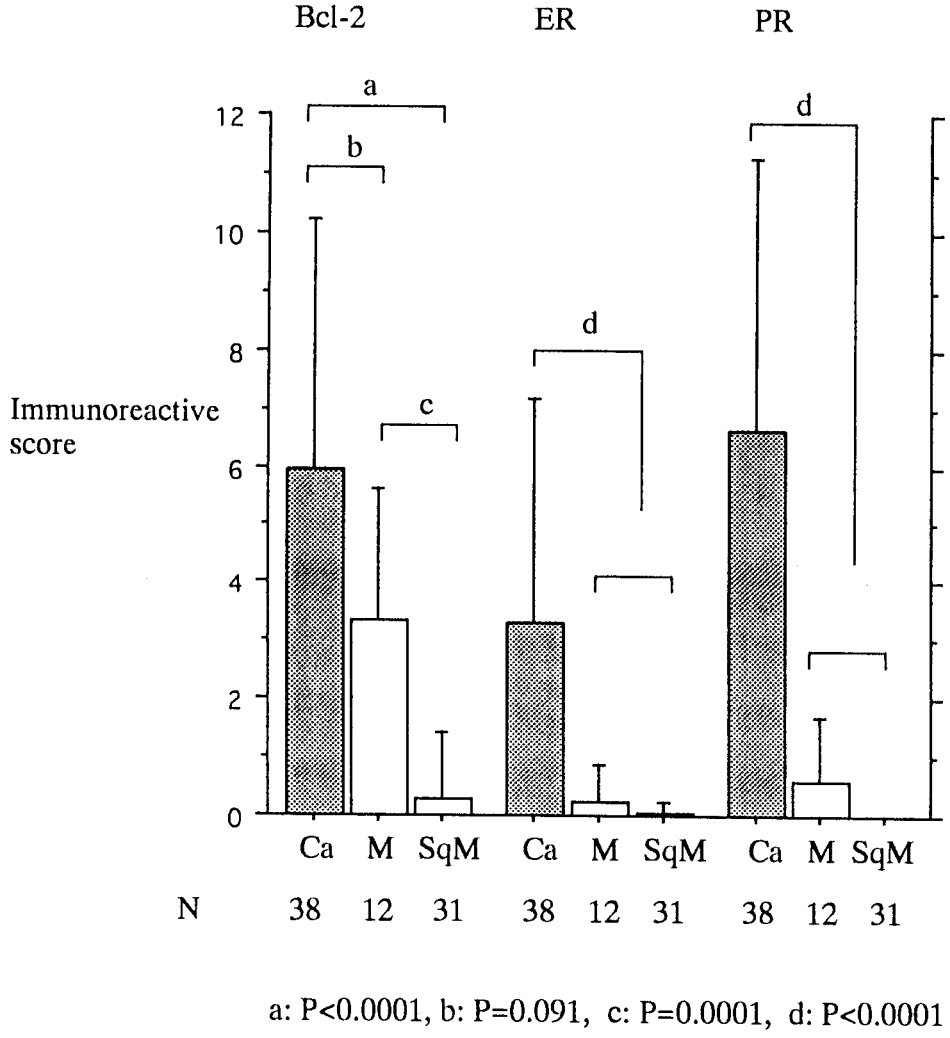

Fig. 3—Immunoreactivity scores for primary tumours and areas of squamous differentiation. CA = carcinomas; M = morules; SqM = Sq-M foci

Table I—Differences in immunopositivity of bcl-2, ER, and PR between primary tumours and areas of squamous differentiation

Immunohistochemistry		Squamous areas					
		Sq-M foci (n=31) (%)			Morules (n=12) (%)		
Primary tumour (n=38)	Squamous areas (n=43)	bcl-2	ER	PR	bcl-2	ER	PR
Positive	Positive	3 (9·7)	1 (3·2)	0	10 (83·3)	2 (16·7)	3 (25)
Positive	Negative	24 (77·9)	17 (54·8)	25 (80·6)	2 (16·7)	4 (33·3)	8 (66·7)
Negative	Negative	4 (12·9)	13 (41·9)	6 (19·4)	0	6 (50)	1 (8·3)
Negative	Positive	0	0	0	0	0	0

Squamous areas include both Sq-M foci and morule lesions. Sq = squamous; Sq-M = squamous metaplasia; ER = oestrogen receptor; PR = progesterone receptor; n = number of cases.

positivity in morules. There were no cases where the converse was observed.

Ki-67 immunoreactivity was clearly limited to nuclei, showing a heterogeneous distribution (Figs 1e and 2e). As shown in Table II, the Ki-67 LIs of tumours were significantly higher than those of areas of squamous differentiation (P<0·0001).

Morphological changes of carcinomas after hormone therapy

The eight cases receiving hormone therapy were subdivided into two groups, good response [therapeutic efficacy (TE) = 3 or 4] and poor response (TE = 1 or 2), with four cases in each.

Table II—Comparison of Ki-67 labelling indices between primary tumours and areas of squamous differentiation

		Ki-67 LI		
	n	*m* ± SD	Range	*P*
Primary tumours	38	27·4 ± 2·1	2·7–58·7	
Squamous areas	43	1·8 ± 0·3	0–7·5	<0·0001

Squamous areas include both Sq-M foci and morule lesions; *n* = number of cases; *m* ± SD = mean ± standard deviation.

In the good response group, tumour cells gradually changed their morphological appearance, with alteration of nuclear arrangement from multiple integrated to mono-layers and an increase in eosinophilic cytoplasm, resulting in reduced cellularity and a decreased N/C ratio, during the period of therapy. The nuclear shape in most tumour cells also changed from spindle or club-like to oval, although atypical structures and nucleoli persisted (Figs 4a and 4f). Areas of squamous differentiation were found in nine (four Sq-M and eight morule foci) of 11 biopsy specimens before hormone therapy and in 27 (nine Sq-M and 20 morule lesions) of 31 samples after treatment.

In the poor response category, although tumour cellularity was slightly decreased by hormone therapy, morphological changes were relatively minor.

Normal endometrial glands frequently showed marked atrophic changes with cystic dilatation and fibrotic change in stromal elements during hormone therapy.

Change in immunoreactivity scores for bcl-2, ER and PR and Ki-67 LIs after hormone therapy

Data in the eight cases are summarized in Table III.

Strong PR immunoreactivity in first biopsy specimens was found in all cases investigated, with the exception of case 6, while in most cases it disappeared immediately on treatment without tumour cell maturation. Positive linkage between decrease in bcl-2 immunoreactivity and reduced Ki-67 LIs was predominantly found in the good response group. In contrast, no apparent change in ER immunoreactivity was evident in either group (Fig. 4).

The average values for each parameter in the eight cases are shown in Table IV. A marked decrease in the bcl-2 and PR scores and Ki-67 LIs was caused by hormone therapy, the differences being significant ($P = 0{\cdot}024$, $P = 0{\cdot}024$, and $P = 0{\cdot}012$, respectively). There was no apparent alteration in ER scores. In contrast, no significant changes in the scores for bcl-2, ER, and PR in areas of squamous differentiation were noted, with the exception of Ki-67 LIs.

A close correlation ($r = 0{\cdot}739$, $P = 0{\cdot}039$) was found between the suppression rate of bcl-2 (% bcl-2) and the therapeutic efficacy, while no association was noted for the % ER ($r = 0{\cdot}122$, $P = 0{\cdot}773$), % PR ($r = 0{\cdot}591$, $P = 0{\cdot}163$) or % Ki-67 ($r = 0{\cdot}649$, $P = 0{\cdot}082$).

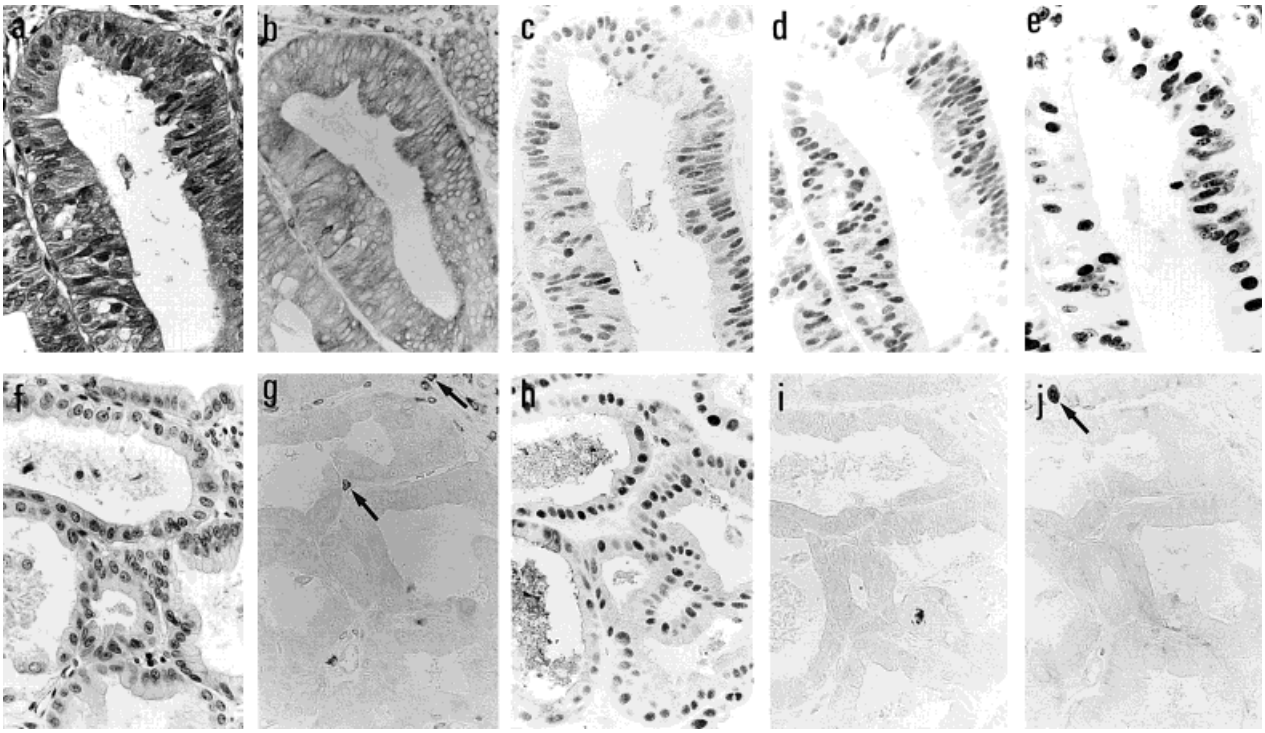

Fig. 4—Morphological and immunohistochemical changes of tumour cells due to progesterone therapy in case 1. (a–e) First biopsy specimen. (f–j) Biopsy taken after 23 weeks of medroxyprogesterone acetate treatment. (a, f) H & E staining. Immunohistochemistry for bcl-2 (b, g), ER (c, h), PR (d, i), and Ki-67 (e, j). A marked decrease in immunoreactivity for bcl-2 (g, strong immunoreactivity in lymphocytes indicated by arrows), PR (i), and Ki-67 (j, single tumour cell immunolabelled, indicated by an arrow) is demonstrated, correlating with morphological changes of tumour cells. ER positivity is still observed after hormone therapy (h)

Table III—Summary of the relation between hormone therapy and immunoreactivity of bcl-2, ER and PR and Ki-67 LIs in endometrial carcinomas

Case No.	Age	Tumour grade	Follow-up time (weeks)	First biopsy (A)* Score bcl-2	First biopsy (A)* Score ER	First biopsy (A)* Score PR	First biopsy (A)* Ki-67 LI (%)	Hormone therapy (B)* Score bcl-2	Hormone therapy (B)* Score ER	Hormone therapy (B)* Score PR	Hormone therapy (B)* Ki-67 LI (%)	Suppression rate (SR) (1 − B/A) % bcl-2	% ER	% PR	% Ki-67	Therapeutic efficacy (TE)
1	30	G1	23	4	12	12	58·7	1	10·7	0	10·2	0·75	0·11	1	0·83	4
2	41	G1	16	12	9	12	24·5	0·7	9	0·7	2·7	0·94	0	0·94	0·89	4
3	37	G1	4	12	6	12	14·1	4	2	2	2·1	0·67	0·67	0·83	0·85	3
4	30	G1	44	12	12	12	7·3	8·5	8·3	12	4·3	0·29	0·31	0	0·42	1
5	28	G1	15	12	8	12	42·8	2	5·2	0·4	8·7	0·83	0·35	0·97	0·8	4
6	25	G1	18	0	1	0	33·9	1·8	8·3	0	13·7	0†	0‡	§	0·6	2
7	28	G1	20	12	4	9	23	7	6·3	0	4·4	0·42	0‡	1	0·8	1
8	33	G1	6	12	8	12	69·8	3·5	6·5	3·5	42·3	0·71	0·19	0·71	0·39	2

ER = oestrogen receptor; PR = progesterone receptor; Ki-67 LIs = Ki-67 labelling indices.
*Average values of several specimens obtained before or after treatment.
†Suppression rate could not be calculated because the first biopsy specimen was negative.
‡Negative suppression rate (<0).
§Negative immunoreactivity in both the first biopsy and hormone therapy specimens.

Table IV—Changes in the immunoreactive scores for bcl-2, ER and PR and of Ki-67 LI after hormone therapy

	Carcinoma lesions ($n=8$)			Squamous areas ($n=8$)		
	First Bx	HRT	P	First Bx	HRT	P
bcl-2	9·5 ± 4·8	3·6 ± 2·8	0·024	2·3 ± 2·4	2·1 ± 2·6	NS
ER	7·5 ± 3·8	7·0 ± 2·7	NS	0·9 ± 1·0	0·6 ± 1·0	NS
PR	10·1 ± 4·2	2·3 ± 4·1	0·024	0·8 ± 1·0	0·3 ± 0·9	NS
Ki-67 LI	34·3 ± 21·7	11·1 ± 13·3	0·012	1·6 ± 1·7	1·1 ± 2·9	0·012

Squamous areas include both Sq-M foci and morule lesions. ER = oestrogen receptor; PR = progesterone receptor; Ki-67 LIs = Ki-67 labelling indices; Bx = biopsy; HRT = hormone therapy; NS = not significant; n = number of cases.

DISCUSSION

The present study clearly demonstrated a negative correlation between bcl-2 immunoreactivity and squamous differentiation within endometrial carcinomas, accompanied by a loss of ER and PR immunoreactivity and a decrease in the Ki-67 LI. Sq-M foci, morphologically resembling differentiated keratinocytes, are generally considered to represent metaplasia of tumour cells. In normal squamous-type epithelia, bcl-2 expression is limited to basal cells and is completely negative in suprabasal cells, suggesting that bcl-2 may play an important role in temporary suppression of cell death coupled with terminal differentiation, through either regulating intracellular Ca^{2+} levels or modulating the effect of endonuclease.[13]

Benito *et al.*[14] demonstrated that down-regulation of bcl-2 appears to be part of a differentiation pathway, possibly serving to facilitate the apoptotic response in the HL-60 promyelocytic cell line. Krajewski *et al.*[15] also reported high levels of bcl-2 immunostaining in neuroblastomas, but much less intense immunoreactivity in more highly differentiated ganglionic cells in ganglioneuroblastomas and ganglioneuromas, suggesting that down-regulation of bcl-2 occurs during terminal differentiation. We can therefore conclude from the present data that the down-regulation of bcl-2 expression in squamous differentiation within endometrial carcinomas may be closely linked with a form of tumour cell differentiation. This conclusion is supported by the decreased immunoreactivity scores for ER and PR and Ki-67 LI values in Sq-M foci and morules, again reflecting terminal differentiation. Given their intermediate status for bcl-2 expression between primary tumours and Sq-M foci, the morules may represent an immature differentiation phenotype, in line with morphological findings. However, considering the occasional finding of areas of squamous differentiation in bcl-2 negative carcinomas, alteration of bcl-2 expression may not be essential for differentiation to occur.

Our previous results indicate that in endometrial carcinomas, bcl-2 expression in tubular components is significantly higher than in solid tumour areas, in negative association with histological grading,[6] supporting the evidence of a close linkage between its expression and the degree of differentiation.[13] Chan *et al.*[16] also demonstrated down-regulation of bcl-2 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 neoplasia. In the present study, the finding of squamous differentiation predominantly in tubular tumour components generally positive for bcl-2 indicates that expression of this proto-oncogene may be closely related to the multidifferentiation potential of tumour cells. Thus, its loss may occur either with squamous differentiation or adoption of a more malignant phenotype (dedifferentiation).

Cyclic bcl-2 expression in normal endometrial glandular cells has been noted,[17] with Gompel *et al.*[18] documenting that immunoreactivity peaks at the end of the proliferative phase and disappears at the onset of secretory activity, suggesting up-regulation by oestradiol and down-regulation by progesterone in the normal physiological homeostasis. Teixeira *et al.*[19] reported that E2 depletion in the oestrogen receptor-positive MCF-7 human breast cancer cell line results in a marked decrease in bcl-2 expression, suggesting that this latter is involved in oestrogen promotion of resistance to chemotherapeutic drugs. The available data thus strongly indicate a direct or indirect linkage between bcl-2 expression and ovarian hormones.

Successful control of endometrial carcinomas is usually dependent on total hysterectomy, with treatment of locally recurrent or initially inoperable carcinomas by hormone therapy or irradiation. The principal variable governing the response of endometrial carcinomas to progesterone therapy is the availability of PR and it is now established that the oral route of administration may require 12 weeks to produce a response.[20] In the present study, morphological changes of tumour cells, resembling those seen in the secretory phase, were already found in the good response carcinomas (TE = 4; cases 1, 2, and 5) within a few weeks of treatment, correlating with loss of bcl-2 immunoreactivity and low Ki-67 LIs. The results suggest that in endometrial carcinomas, bcl-2 expression may be down-regulated by progesterone, probably through tumour cell maturation and that this protein may therefore be useful as a marker for the efficacy of progesterone therapy. In addition, the finding that bcl-2 immunoreactivity in areas of squamous differentiation was not altered by treatment suggests that different pathways may exist between squamous differentiation signal and the maturation

effect by progesterone. Further studies are clearly warranted. The significant decrease in the Ki-67 LIs in these areas may be associated with suppression of proliferative activity in primary lesions. In contrast, the rapid disappearance or decrease in PR immunoreactivity caused by hormone treatment may be simply a reflection of the negative feed-back system between progesterone and PR expression in tumour cells. The fact that ER immunoreactivity was not altered by the administration of progesterone indicates that the receptor expression itself is not affected by progesterone.

In conclusion, the present study provides strong evidence that in endometrial carcinomas, down-regulation of bcl-2 expression may be closely linked with squamous differentiation and associated with changes in ER and PR expression, as well as cell proliferation. In addition, bcl-2 levels are markedly decreased by progesterone therapy in good response tumours, suggesting that this parameter may be a clinically useful marker of hormone therapy effects on endometrial carcinomas.

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