BCL-2 IMMUNOREACTIVITY INCREASES WITH SEVERITY OF CIN: A STUDY OF NORMAL CERVICAL EPITHELIA, CIN, AND CERVICAL CARCINOMA

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SUMMARY

The presence of the BCL-2 protein, a marker for inhibition of programmed cell death, was studied in a series of routinely processed cervical tissues, consisting of normal endocervical (n=40) and ectocervical epithelium (n=27), squamous metaplastic epithelium (n=30), CIN (cervical intraepithelial neoplasia) lesions (n=32), and cervical carcinomas (n=13). BCL-2 was strongly expressed in the basal cell compartment of normal ectocervical squamous epithelium and in nearly all reserve cells, while in endocervical columnar cells it was moderately expressed. In immature squamous metaplastic epithelium, BCL-2 expression varied. Half of the cases showed only basal cell staining, while the other half showed staining also in suprabasal layers. BCL-2 could be detected in all premalignant lesions, showing a striking increase in the number of positive cells with increasing severity of CIN, in combination with a mild increase in staining intensity. All adenocarcinomas were positive (n=5), while five of eight squamous cell carcinomas expressed BCL-2. Based on these results, it is hypothesized that both the larger number of cells staining with BCL-2 in higher grades of CIN and the increase in staining intensity imply an increasing protection of these neoplastic conditions against programmed cell death. This protection facilitates not only continuing proliferation, but also the induction of genetic instability in dysplastic epithelial cells; it may thus reflect the greater capacity of the more severe CIN lesions to evolve into cervical carcinoma.

KEY WORDS—BCL-2; immunocytochemistry; dysplasia; prognosis; apoptosis; cervix; carcinoma

INTRODUCTION

Expression of the BCL-2 gene, located on chromosome 18, was first described in follicular B-cell lymphomas containing the t(14;18) translocation.1,2 The BCL-2 proto-oncogene codes for a protein localized mainly in the mitochondrial membrane, but also found in the nuclear membrane.3 The putative role of the BCL-2 protein is to extend cell survival, by protecting the cell against programmed cell death (apoptosis) without affecting cell proliferation.4 Lymphoid cells with the t(14;18) translocation have been shown to accumulate because they are not susceptible to apoptosis, but a number of studies have shown that expression of BCL-2 may also be demonstrated in cells without the t(14;18) translocation.5 In these apparently normal cells, which often comprise proliferative stem cell compartments of tissues or cells with a prolonged lifespan, the BCL-2 protein is also involved in the inhibition of programmed cell death.

Studies investigating the presence of BCL-2 protein in embryonal and normal non-lymphoid tissues, such as epithelial, neural, mesenchymal, and endocrine tissues, also support the role of this protein in tissue homeostasis.6,7 In a recent study of breast carcinomas,8 BCL-2 was shown to be present in more than 80 per cent of the cases and was co-expressed with the oestrogen receptor. In lung carcinomas,9 BCL-2 expression is suggested to be of prognostic relevance. Strikingly, however, the BCL-2-negative malignancies have a poor prognosis. In the normal cervix, Hockenbery et al.9 did not observe the BCL-2 protein in any of the cell types, although its expression could be expected on the basis of studies in other normal epithelia, such as skin, intestine,7 breast,8 and bronchi.9 To our knowledge, there are no published studies concerning BCL-2 protein expression in CIN lesions or cervical carcinoma. We therefore examined BCL-2 expression in a variety of cervical tissues, including normal cervical epithelium, CIN lesions, and cervical carcinomas.

MATERIALS AND METHODS

Formalin-fixed and paraffin-embedded tissue specimens were used in this study. Excision biopsies from the cervix were taken from hysterectomy specimens removed for benign conditions, which served as controls (n=16). For CIN lesions, diathermy loop excision specimens were taken from women suspected of CIN on the basis of cytological examination. These consisted of ten cases of CIN I, nine of CIN II, and 13 of CIN III. Tissue samples from eight squamous cell carcinomas and five adenocarcinomas were taken from radical hysterectomy specimens. In some cases, more than one type of epithelium was investigated in a tissue specimen. In this way, 27 cases with ectocervical non-keratinizing epithelium, 40 with endocervical columnar epithelium, 22 with reserve cells, 8 of immature squamous metaplasia, and 22 of mature squamous metaplasia were seen.
The histological diagnosis was made on haematoxylin and eosin (H & E) stained slides according to current standard criteria.

**Immunostaining**

For immunocytochemistry, 2 μm thick sections were cut from representative paraffin blocks. The sections were deparaffinized in xylene and rehydrated. After blocking of the endogenous peroxidase activity with 0.2 per cent H₂O₂, the sections were incubated in citrate buffer (0.01 M; pH 6.0) for two cycles of 5 min in a microwave oven (Panasonic), at 700 W for antigen retrieval. For localization of the BCL-2 protein, the sections were thereafter incubated for 60 min at room temperature using a 1:50 dilution of the monoclonal antibody 124 mAb (DAKO A/S, Glostrup, Denmark), visualized by the indirect avidin–biotin procedure (High Performance Multi Link Kit, BioGenex, San Ramon, U.S.A.) using diaminobenzidine (DAB) as the substrate chromogen. The immunostained slides were counterstained for 1 min with haematoxylin. Normal lymphocytes infiltrating the stroma of the cervical lesions represented an internal positive control for BCL-2 immunostaining. Further positive controls consisted of lymph node tissue with reactive follicular hyperplasia, in which the germinal centre cells of the lymphoid follicle were negative and most of the follicle mantle zone cells were positive, as described elsewhere.¹

**Evaluation of immunostaining results**

All H & E and immunostained slides were independently reviewed by two observers (BT, FS). In cases of discrepancy, the slides were reviewed together and consensus was reached in all cases. In all positive cases, immunoreactivity was restricted to the cytoplasm. The percentage of positive cells in the respective cell compartments of each type of epithelium and in the cervical carcinomas was determined semi-quantitatively in relation to the total number of cells in the epithelial compartment or carcinoma. The number of positive cells was scored using five categories: <5 per cent, negative (category 1); 5–25 per cent (category 2); 25–50 per cent (category 3); 50–75 per cent (category 4); and 75–100 per cent (category 5). The staining intensity was graded as strong, moderate, weak, or very weak.

**RESULTS**

The results of the study are represented schematically in Figs 1A and 1B and depicted in Fig. 2. The following are the most salient features.

**Normal cervical epithelium (Fig. 1A)**

The basal cell compartment of the ectocervical epithelium in all tissue specimens usually showed strong BCL-2 immunoreactivity in practically all cells, the suprabasal epithelial layers staining less frequently and less strongly (Fig. 2A). In the direct vicinity of the squamocolumnar junction, basal cell staining was usually less than in other parts of the ectocervix. Overlying cell compartments, however, displayed very weak immunoreactivity (Fig. 2b), the same phenomenon being also observed in foci showing chronic cervicitis, where lymphocytes infiltrated the epithelium. In three cases showing parakeratosis, strong immunoreactivity was observed in the basal cell compartment (Fig. 2c), while moderate staining was observed in several cells of the parabasal layer of the ectocervix, with the exception of the parakeratotic layer, which was negative.

In 22 cases of endocervix, BCL-2-positive reserve cells, staining strongly and with little variation, were identified beneath the columnar cells (Fig. 2d). All 40 tissue fragments containing endocervical columnar cells showed usually weak immunoreactivity for BCL-2 in approximately 75 per cent of cells. Staining was granular, generally in the apical part of the columnar cells, although in some cases moderately strong perinuclear immunostaining was also observed. In a few cases, foci of columnar cells showed more intense BCL-2 staining.

BCL-2 immunoreactivity in immature squamous metaplasia was highly variable. In four out of eight cases, basal and intermediate cell compartments stained weakly, while in the other four cases, moderate staining was seen in the basal cell compartment only (Fig. 2e), the overall intensity of immunostaining being usually greater than in those cases showing BCL-2 staining in the higher cell compartments.

The 22 tissue fragments showing mature squamous metaplasia displayed strong immunoreactivity with the BCL-2 antibody in practically all cells of the basal cell compartment (Fig. 2f). In ten cases, dispersed, weak staining was seen in the parabasal cell layer and in four cases some scattered cells stained very weakly in the intermediate cell layer. BCL-2 was not demonstrated in the superficial cell compartment.

**Subepithelial tissues**—Most lymphocytes in the stromal compartment displayed strong perinuclear immunoreactivity. Smooth muscle cells of small arteries and stromal fibroblasts showed weak, variable staining, while endothelial cells were occasionally weakly immunodecorated.

Normal cervical epithelia in the control cases stained identically to normal epithelia found in cervical tissue fragments containing CIN or cervical carcinoma.

**Cervical intraepithelial neoplasia (CIN; Fig. 1B)**

**CIN I**—This type of epithelium was seen in ten tissue fragments. In all cases, weak immunoreactivity with the BCL-2 antibody was observed in approximately 50 per cent of the cells in the basal cell compartment (Fig. 2g). The other cell compartments were invariably negative.

**CIN II**—The nine cases in which this epithelium was found all displayed immunoreactivity. About 75 per cent of cells in the basal cell compartment stained moderately with the BCL-2 antibody. In one case, the intermediate cell compartment showed weak staining in a minority of cells, while in another case, both intermediate and superficial cell compartments were stained.
Fig. 1—Schematic representation of BCL-2 expression in normal cervical epithelia (A), CIN lesions, and cervical carcinomas (B). The average number of cells staining in the respective cell compartments is divided into five categories and is indicated by the height of the bars. The shading in each bar indicates the relative staining intensity, with black bars denoting strong staining and white bars very weak staining. ISM = immature squamous metaplastic epithelium; MSM = mature squamous metaplastic epithelium; co = columnar cells; re = reserve cells; b = basal cell compartment; pb = parabasal cell compartment; i = intermediate cell compartment; s = superficial cell compartment; sc = squamous cell carcinoma; ad = adenocarcinoma.

**CIN III**—In all 13 lesions, 90 per cent of the cells in the basal cell compartment were moderately immunostained by the BCL-2 antibody. In five cases, about 50 per cent of the cells in the intermediate cell compartment stained weakly, while in five other cases, BCL-2 was detected throughout the full thickness of the lesion (Fig. 2a). With increasing severity of CIN, the percentage of positive cells in each compartment increased, as did the staining intensity.

**Carcinomas (Fig. 1B)**

The group of squamous cell carcinomas comprised six non-keratinizing and two small cell non-keratinizing carcinomas. Three of the non-keratinizing squamous cell carcinomas showed moderate immunoreactivity with the BCL-2 antibody in approximately 50 per cent of the tumour cells, with no apparent compartmentalization of staining (Fig. 2i), while the other three cases were negative (Fig. 2j). The small cell non-keratinizing carcinomas displayed strong immunoreactivity in practically all cells in the basal compartment, with a few cells staining weakly in the other compartments. BCL-2 was expressed in all five adenocarcinomas, although the staining intensity varied from weak to moderate, irrespective of the grade of differentiation. Approximately 85 per cent of the tumour cells were decorated by the BCL-2 antibody (Figs 2k and 2l).

**DISCUSSION**

The present study indicates that BCL-2 protein is immunohistochemically detectable in the stem cell compartment of normal cervical epithelia, in CIN, and in most cervical carcinomas. In contrast to a previous study of Hockenbery et al.,7 who used a different immunoreagent, expression of this protein was observed in the basal cell layer of normal ectocervical epithelium. This reflects the progenitor cell role of basal cells, which require the protection of BCL-2 against apoptotic cell death to ensure survival of the entire epithelium. Its absence in the suprabasal layers indicates that BCL-2 is not required during completion of the differentiation...
process, which has a duration of approximately 4 days. The ubiquitous presence of BCL-2 protein in reserve cells again reflects their stem cell function; BCL-2 expression maintains the integrity of the basal cell compartment by imparting an extended lifespan to the reserve cells.

In a few cases of normal ectocervical squamous epithelium, weak BCL-2 staining was recognized in parabasal and intermediate cell compartments, often associated with chronic inflammation in which cell turnover is increased. Since the epithelial cells above the basal layer have the morphological characteristics of basal cells, BCL-2 expression could correlate with these features. The observation of lower levels of basal expression in the direct vicinity of the squamocolumnar junction, combined with BCL-2 expression in the intermediate epithelial layers, may be explained on the basis of increased cell turnover. In cases of parakeratosis, BCL-2 expression was seen reaching into the intermediate cell layer. This may be explained either as suppression of the normal differentiation process by the BCL-2 protein, or by BCL-2 protection of these intermediate cells against
programmed cell death, resulting in BCL-2 accumulation in higher epithelial cells. Some columnar cells displayed high levels of immunoreactivity, especially where reserve cells were present. This possibly reflects the fact that reserve cells are the progenitor cells of the columnar epithelium. A similar hypothesis has also been suggested on the basis of keratin phenotyping studies in the cervix. We suggest that the columnar cells with the highest BCL-2 expression levels are not yet fully differentiated, or have just evolved from the underlying reserve cells.

In immature squamous metaplasia, BCL-2 was weakly expressed in the intermediate cell compartment. In comparison, staining in mature squamous metaplasia was mainly restricted to the basal cell compartment and therefore displayed features partly characteristic of normal ectocervical squamous epithelium. BCL-2 expression in immature squamous metaplasia reflects the reserve cell features of this epithelium, a feature also found in keratin studies of immature and mature squamous metaplasia.

With the progression of CIN, increasing levels of BCL-2 immunoreactivity were observed. First of all, the number of BCL-2-positive cells increased. Secondly, immunostaining became increasingly apparent in the higher cell compartments. Thirdly, the intensity of immunostaining seemed to increase in the respective epithelial layers with increasing severity of the lesion. Thus the increasing severity of CIN lesions is reflected by the BCL-2 expression levels. We hypothesize that low levels of BCL-2 in CIN I indicate that in these lesions this proto-oncogene imparts only limited protection against cell death and thus only a small number of CIN I lesions will progress. The same explanation may be proposed for the CIN II lesions, which display a slightly increased BCL-2 immunoreactivity in comparison with CIN I. In CIN III, BCL-2 protein extends into the higher layers of the epithelium, guarding this epithelium from apoptosis, allowing CIN III lesions to persist and consequently impairing to them a feature which will enable them to develop into cervical carcinoma. This may also hold true for the CIN II lesions with extensive expression of BCL-2, which may reflect their progressive capacity. The higher level of BCL-2 protein in the higher degrees of CIN may indicate that an increased proliferative capacity of this epithelium is of fundamental importance in the development of a cervical carcinoma and that protection of epithelial cells against programmed cell death may also play an important role.

Another important factor may be the introduction of stable genetic aberrations in a certain fraction of tumour cells, resulting in genetic instability of this cell population. Such genomic changes may then induce a more aggressive, invasive phenotype in a small fraction of the affected cells. Accumulation of mutations is well known in more advanced carcinomas. In these cases, the large number of mutations could influence the synthesis of the BCL-2 protein. Furthermore, in BCL-2-negative carcinomas, protection against apoptosis may not be of fundamental importance in maintaining the neoplasm; in these carcinomas, proliferative capacity may be a more important factor.

The observation that BCL-2 expression occurred in only half of the cases of squamous cell carcinoma of the cervix is difficult to explain. In a study on primitive peripheral neuroectodermal tumours, not all cases were immunoreactive for BCL-2, the positive cases showing an unfavourable outcome. In a study of squamous cell carcinomas of the lung, BCL-2 expression indicated a more favourable prognosis; in breast carcinomas, no relationship was observed between BCL-2 expression and tumour stage. It is evident that BCL-2-negative carcinomas have been observed by others, but the prognostic significance of BCL-2 has still to be established.

In summary, our results suggest that BCL-2 proto-oncogene expression in CIN plays a role in the development of cervical carcinoma by protection against apoptosis. Prognostic studies, relating BCL-2 expression to clinical outcome, will be required to test this hypothesis.

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REFERENCES