Progressing Imbalance Between Proliferation and Apoptosis with Increasing Severity of Cervical Intraepithelial Neoplasia

Bram ter Harmsel, M.D. Johan Kuipers, M.D., Ph.D. Frank Smedts, M.D., Ph.D. Marcel Jeunink, C.T. Baptist Trimbos, M.D., Ph.D. and Frans Ramaekers, Ph.D.

Summary: The equilibrium between cell proliferation and protection against apoptosis was studied immunohistochemically using monoclonal antibodies against Ki-67-Ag and bcl-2, respectively, in consecutive sections from normal and metaplastic cervical epithelia and cervical intraepithelial neoplasia (CIN) lesions and cervical carcinomas. A high percentage of Ki-67-Ag positive cells was seen in the parabasal cells of normal ectocervical and mature squamous metaplastic epithelium, although the basal cells were virtually negative. In preneoplastic lesions, however, the basal cells showed high proliferative activity and an increasing frequency of Ki-67-Ag positive cells was observed in the higher epithelial layers with increasing severity of CIN. In squamous cell carcinomas, variable numbers of Ki-67-Ag positive cells were observed and in adenocarcinomas expression increased with the degree of anaplasia. bcl-2 expression was observed only in the basal cells of normal endo- and ectocervix including reserve cells. With increasing severity of CIN, staining intensity and number of bcl-2 positive cells gradually increased. Five of eight squamous cell carcinomas were variably positive. All five adenocarcinomas showed extensive bcl-2 expression. Increased expression of both Ki-67-Ag and bcl-2 with increasing severity of CIN indicates an increasing imbalance between cell proliferation and protection from apoptosis. It is therefore proposed that an increasing proliferative fraction combined with a higher number of cells protected from apoptotic cell death contributes to progression of CIN. This phenotype may identify premalignant lesions with the potential to transform to cervical cancer. Key Words: Ki-67-Ag—bcl-2—Cervical intraepithelial neoplasia—Cervical carcinoma.

Cervical carcinoma originates from cervical intraepithelial neoplasia (CIN) and is usually located at the squamocolumnar junction. With time, many CINs increase in severity and eventually become frankly malignant (1). Low-grade CIN is believed to have a low malignant potential (the risk of progression in cervical carcinoma is low), and most of these lesions are thought to regress spontaneously. High-grade CIN has a greatly increased malignant potential, and according to some investigators (2), this may approach 100% progression to carcinoma. On the basis of pure morphologic criteria, it is not possible to identify CIN lesions that will follow a progressive course or to distinguish them from those that will regress. For this reason, we decided to investigate the reactivity of CIN and cervical carcinomas with panels of antibodies that have been shown to indicate specific features of malignant progression, such as markers of the cell cycle, proto-oncogenes, and tumor suppressor genes.

The monoclonal antibody MIB1 detects Ki-67-Ag, present in cells in all active phases of the cell cycle (G1, S, G2, and M), but is absent in G0 (3,4). The antibody has been shown to be extremely useful in the evaluation of proliferative activity in normal tissues and malignant tumors and has been suggested to be of prognostic value.
(5). An earlier investigation in cervical carcinoma showed that, in all cases, between 10 and 50% of cells showed Ki-67-Ag expression independent of the cell type or tumor grade (6). To examine the cytoplasmic parameters in the progression of CIN, we investigated the expression of Ki-67-Ag in normal, metaplastic, and pre-neoplastic cervical epithelia. The aim of the study was to determine whether differences in Ki-67-Ag expression occurred between normal cervical epithelia and CIN, and also whether Ki-67-Ag expression varies between the various grades of CIN.

Equally important for progression of CIN may be the capacity of dysplastic epithelial cells to withdraw from programmed cell death (apoptosis). bcl-2 is considered to protect cells from apoptosis and is therefore expressed in stem cell compartments and other cells in which protection from cell death is important for tissue homeostasis (7,8). In premalignant lesions, accumulation of bcl-2 product can lead to a prolonged life-span of cells, which may result in an accumulation of genetic aberrations in cells that are highly protected against cell death. For this reason, we investigated bcl-2 expression in CIN and compared the bcl-2 positive fraction with the compartment positive for Ki-67-Ag.

MATERIALS AND METHODS

Formalin-fixed and paraffin-embedded tissue specimens were used from excision biopsies from cervices removed for benign conditions, which served as controls (n = 16). For CIN lesions, diathermy loop excision specimens were used from women with cytologically verified CIN. Specimens consisted of 16 cases of CIN I, 12 cases of CIN II, and 13 cases of CIN III. Tissue samples from eight cervical 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, 29 cases with normal ectocervical nonkeratinizing epithelium, 37 samples with normal endocervical epithelium, 20 cases with reserve cells, 8 cases of immature squamous metaplasia, and 13 cases of mature squamous metaplasia were examined. Histologic diagnosis was established on hematoxylin and cosin (H&E) stained slides.

Immunostaining

For Ki-67-Ag immunocytochemistry with antibody MIB1 (Immunotech, Marseille, France), 2-μm thick sections were cut from representative paraffin blocks, mounted on organosilane-coated slides, and dried overnight. After deparaffination and blocking of endoge-aneous peroxidase activity, the sections were incubated in citrate buffer (0.01M, pH = 6.0) for two consecutive periods of 5 minutes in a microwave oven at 750 W. Between the two periods, the volume of citrate buffer was checked and adjusted when needed. The sections were then incubated with the undiluted antibody MIB1 for 1 hour at room temperature. After subsequent washing steps with phosphate buffered saline (PBS), the peroxidase labeled Steptavidin detection system (BioGenex Laboratories, San Ramon, CA, USA) was applied to the sections. The peroxidase activity was detected with diamine-benzidine HCL (DAB). The sections were counterstained, dehydrated, and mounted with coverslip.

The immunohistochemical staining procedure for bcl-2 was essentially the same as described above for MIB1. The monoclonal antibody 124 mAb (DAKO A/S, Glostrup, Denmark), was diluted 1:50 and the signal visualised by the indirect avidin-biotin procedure (High Performance Multi Link Kit, BioGenex, San Ramon, USA) using DAB as a chromogen.

Positively staining normal lymphocytes that infiltrated the stroma of the cervical lesions represented an internal positive control for bcl-2 immunostaining. In addition, positive controls for MIB1 and bcl-2 consisted of lymph node tissue with reactive follicular hyperplasia. For bcl-2, the germinal center cells of the lymphoid follicle were negative and most of the follicle mantle zone cells were positive, as described elsewhere (8).

Evaluation of Immunostaining Results

All H&E stained and immunostained slides were independently reviewed by two authors (B.T.H., F.S.). In cases of discrepancy, the slides were reviewed together and consensus was reached in all cases. MIB1 staining was in general strong and was evaluated by counting 200 successive nuclei. The MIB1 index was determined by dividing the number of MIB1 staining nuclei by 200 and expressing this as a percentage of the total number of cells counted. In the different types of epithelia, the MIB1 staining index was determined for each epithelial compartment separately. In Figure 1, the average MIB1 index is represented and also the range of the MIB1 index. In some cases, minor cytoplasmic staining was observed, which was recorded separately. bcl-2 showed cytoplasmic staining that was graded as strong, moderate, weak, or very weak. Strong bcl-2 staining was defined as cytoplasmic staining as strong as the most intensely staining lymphocytes in the control slides of a reactive lymph node. Very weak staining for the bcl-2 antibody was defined as staining that was just discernable. Weak and moderate bcl-2 staining were estimated.
KI-67-AG AND bcl-2 IN UTERINE CERVIX

Ectocervical Non-Keratinizing Squamous Epithelium

The Ki-67-Ag, as detected by MIB 1, was prominent in nuclei of cells in the parabasal cell layer (Fig. 2a). An average of 34% of these nuclei stained positively, the range varying between 10 and 60%. Only sporadic nuclei (less than 1%) of the basal cells were positive for Ki-67-Ag. The intermediate and superficial cell compartments were completely negative. bcl-2 was identified in >95% of the basal cells of all specimens (Fig. 2d). In the overlying epithelial layers, a fraction of the cells stained very weakly.

Endocervical Columnar Epithelium

Nuclear staining for Ki-67-Ag was observed in <1 of endocervical columnar cells (Fig. 2b) in 12 of 37 fragments. In these positive cases, the cell cytoplasm also displayed some immunoreactivity. bcl-2 immunoreactivity was observed in approximately 75% of these cells and was usually weak. The other specimens were negative (Fig. 2e).

Reserve Cells

In 2 of 20 specimens with reserve cells, Ki-67-Ag was detected with one specimen showing approximately 20% of nuclei positive and the other cases only showing 1% (Fig. 2b). bcl-2 was detected in nearly all reserve cells in all tissue specimens. Staining was usually moderate (Fig. 2e).

Immature Squamous Metaplastic Epithelium

Of the eight tissue specimens in which squamous metaplastic epithelium was diagnosed, four specimens were from the control group and four specimens were associated with CIN lesions. Ki-67-Ag was expressed in all eight cases. In the control specimens, approximately 5% of the basal cell nuclei were positive (Fig. 2c), whereas in the immature metaplastic epithelium associated with CIN, approximately 15% of nuclei in the basal cell layer were stained. In six cases, up to 7% of nuclei in the intermediate cell compartment stained. In one control case, approximately 10% of the nuclei stained in the superficial cell compartment. bcl-2 staining in immature squamous metaplastic epithelium was variable, with all cases showing weak to moderate cytoplasmic staining of the basal and intermediate cells (Fig. 2f).

Mature Squamous Metaplastic Epithelium

Staining with the antibodies for MIB 1 and bcl-2 was identical to that observed in normal ectocervical nonke-
ratinating squamous epithelium (compare Fig. 2a and 2d).

Cervical Intraepithelial Neoplasia

CIN I: The Ki-67-Ag was expressed in all 16 tissue fragments with CIN I. The percentage of positively staining nuclei varied considerably between the individual cases (Fig. 3a). In the basal cells, nuclear staining varied from 10 to 70% of the cells, with approximately 40% of the nuclei staining in the majority of cases. In 12 cases, staining was noted in 5 to 50% of nuclei of the intermediate cells. Superficial staining was noted in 3 of 16 cases, all showing < 5% positive nuclei.

bcl-2 was weakly immunodetected in an average of 50% of the basal cells only in all tissue specimens (Fig. 3d). There was no correlation between the number of cells staining and intensity of immunostaining between Ki-67-Ag and bcl-2.

CIN II: The 12 tissue specimens with this lesion showed nuclear positivity for Ki-67-Ag in the basal cells,

![Image](Image.png)

**FIG. 2.** Immunohistochemical staining of normal cervix for Ki-67-Ag (A,B,C) and bcl-2 (D,E,F). (A,D) ectocervical non-keratinizing squamous epithelium; (B,E) columnar cells and reserve cells of the endocervix, (C,F) immature squamous metaplasia. Original magnifications: (A,B,D,E) ×800, (C,F) ×400.

**FIG. 3.** Immunohistochemical staining of CIN I-III for Ki-67-Ag (A,B,C) and bcl-2 (D,E,F). (A,D) CIN I; (B,E) CIN II; (C,F) CIN III. Original magnification: ×425.

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with an average of 40% of the nuclei staining, exhibiting a variation between 20–70%. In all cases, the intermediate cells were stained, with the number of positive nuclei varying between 20 and 70% (average 33%). In the superficial compartment, between 1 and 70% of the nuclei stained for the Ki-67-Ag, with an average of 13% (Fig. 3b), with only one negative case.

When examined for bcl-2, the basal cells of CIN II showed moderate expression in all tissue specimens (Fig. 3e). In 2 fragments, weak and moderate staining was seen in the intermediate cells, whereas one case stained throughout the full epithelial thickness. There was no evident quantitative relationship between the staining patterns of Ki-67-Ag and bcl-2, meaning that intense staining with one antibody did not mean that high or low levels of the other antigen were present.

CIN III: All 13 tissue fragments displayed immuno-reactivity for Ki-67-Ag. In the basal cells, an average of 45% of the nuclei stained varying between 1 and 70% (Fig. 3c). In the intermediate and superficial cells, 12 of 13 cases showed expression in 1 to 70% of the nuclei with an average of 40 in the intermediate and an average of 24% in the superficial cells.

In all CIN III lesions, 95% of the basal cells were moderately immunodecorated by the bcl-2 antibody (Fig. 3f). In the intermediate cells, 11 fragments showed weak immunoreactivity in approximately 60% of cells. In 5 lesions, there was also very weak bcl-2 immunoreactivity in the superficial layer in approximately 30% of the cells.

Three lesions showed high levels of Ki-67-Ag and bcl-2 in all cell layers.

Carcinomas

This group comprised eight nonkeratinizing squamous cell carcinomas and five adenocarcinomas of the cervix. The Ki-67-Ag antibody decorated all carcinomas with an average of 40% of all nuclei staining, ranging from 5 to 70%, which was similar for both tumor types (Fig. 4a and 4b). Ki-67-Ag expression increased with grade of the adenocarcinoma.

bcl-2 was expressed in all adenocarcinomas, showing weak to moderate staining in approximately 90% of malignant cells (Fig. 4d). The squamous cell carcinomas showed weak bcl-2 immunoreactivity in five of eight cases, with an average of 38% of the cells staining (Fig. 4c).

DISCUSSION

In this study, we examined the balance between the proliferative and apoptotic cell fractions of normal cervical epithelia, CIN lesions, and cervical carcinomas. Cell proliferation was measured using the Ki-67-Ag as a marker, whereas bcl-2 expression was taken as a marker of protection against apoptosis.

The high Ki-67-Ag expression levels seen in nuclei of the parabasal cells of ectocervical epithelium reflect the fact that these cells harbor the highest growth fraction, which confirms earlier observations using bromodeoxyuridine (BrdU) as an indicator of cell proliferation (9). In contrast, as shown in this and other studies, the basal
cells show a relatively low growth fraction. Surprisingly, however, a recent study by Raju (10) showed expression of the proliferation marker PCNA in both the basal and parabasal cells of the ectocervix. This discrepancy between Ki-67-Ag and PCNA may be explained by the fact that the half-life of PCNA exceeds 20 hours, which could result in some staining of nuclei in the G₂ phase in the basal cells (11).

With respect to expression of the Ki-67-Ag in basal and parabasal cell nuclei, a striking difference was observed in immature squamous metaplasia and CIN as compared with normal ectocervical and mature squamous metaplastic epithelium. The former epithelia all show pronounced proliferative activity in the basal cells as concluded from Ki-67-Ag expression, whereas in the latter, the proliferative compartment is limited to the basal cells only. Also, an increase in Ki-67-Ag expression in the successive grades of CIN was seen. Both the number of Ki-67-Ag positive nuclei and the extent of staining in the superficial layers increased with progression of CIN. The increase in the average number of Ki-67-Ag positive nuclei indicates that the growth fraction in the various CIN lesions may be a useful parameter capable of indicating the severity of CIN.

However, a few cases of low-grade CIN showed higher proliferative fractions than the average proliferative fraction of high-grade CIN. Equally, some cases of high-grade CIN lesions showed lower numbers of Ki-67-Ag positive nuclei than observed in most CIN I lesions. It is tempting to speculate that lesions morphologically classified as CIN I with high levels of Ki-67-Ag may have a higher progressive potential than CIN III with a low proliferative fraction. Therefore, it would be equally tempting to speculate that Ki-67-Ag expression could be used as an indicator for the progressive potential of a CIN lesion. However, without adequate retrospective or prospective studies these comments remain speculative. In this context, it would be extremely interesting to investigate whether Ki-67-Ag can act as a classifier in cervical smears, particularly in cases in which low-grade CIN is diagnosed.

In addition, it is interesting to note that immature squamous metaplastic lesions associated with CIN showed higher levels of Ki-67-Ag staining than immature squamous metaplastic lesions seen in controls. This observation could be an early indication that increased proliferation, as indicated by the extent of Ki-67-Ag expression in this immature squamous epithelium, precedes the morphologic changes characteristic of CIN. Thus, this epithelium may not follow the usual path of differentiation to mature metaplastic epithelium as observed in mature metaplasia of control specimens, but will develop into CIN. Also of particular interest is the observation that the level of Ki-67-Ag expression in immature squamous metaplasia is considerably lower than in the low-grade CIN, meaning that these two types of epithelium may be easily separated on basis of the levels of Ki-67-Ag expression.

bcl-2 expression in the basal cells of the ectocervix reflects the stem cell function of this compartment. Apparently, bcl-2 is required for protection of these stem cells against apoptotic cell death, thereby maintaining epithelial homeostasis. The presence of bcl-2 in these basal cells and in the reserve cells and columnar cells of the endocervix, which are cell compartments with low proliferative capacity as evidenced by the few Ki-67-Ag positive cells, is apparently necessary for maintaining these epithelia with stem cell characteristics.

bcl-2 expression increases with increasing severity of CIN, and in this way an extending cell compartment arises that combines both Ki-67-Ag and bcl-2 expression. However, lesions with high levels of Ki-67-Ag expression do not always display high levels of bcl-2 expression or vice versa, which suggests that proliferative activity and apoptosis may sometimes be relatively independent factors in maintaining CIN. In cases in which both markers are intensely expressed, protection from apoptotic cell death and an increased growth fraction have a synergistic effect on the accumulation of dysplastic cells. In these lesions, the accumulation of genomic aberrations is likely to occur because cells that acquire irreversible genetic damage during DNA replication are no longer prone to apoptosis. When at this stage of the malignant process the function of p53 is impaired, the scene is set for transformation to carcinoma. In a previous study (12), we showed that most CIN lesions exhibit low levels of p53 expression, while 60% of carcinomas show extensive p53 immunostaining, indicating a late role of p53 mutations in the carcinogenic process of the cervical epithelium.

Future studies will need to reveal whether the combination of these markers may be diagnostically helpful in determining the severity of CIN, in particular in cervical smears containing only a limited number of cells. Furthermore, the staining patterns of these markers in CIN and carcinomas may provide important information on the progressive potential of CIN and on the prognosis in patients with cervical carcinoma.

REFERENCES


