Expression of the BCL-2 protein in normal and dysplastic bronchial epithelium and in lung carcinomas

C Walker¹, L Robertson¹, M Myskow² and G Dixon²

¹Clatterbridge Cancer Research Trust, J K Douglas Cancer Research Laboratory, Clatterbridge Hospital, Bebington, Wirral L63 4JY; ²Department of Histopathology, Broadgreen Hospital, Liverpool L14 3LB, UK.

Summary  Although expression of the bcl-2 protein has been investigated in a number of non-haematological malignancies, little is known of its distribution in premalignant lesions. Expression of bcl-2 was investigated immunohistochemically in archival biopsies of normal (n = 8) and dysplastic bronchial epithelium (n = 56) and in 31 bronchial resection margins and their corresponding carcinomas. All dysplasias had lost the prominent basal staining pattern seen in histologically normal epithelium. Two were negative and six had occasional basal positive cells. In 37 cases up to 66% of the epithelial cells throughout the full epithelial thickness were bcl-2 positive with weak to moderate staining intensity. In 11 cases, all severe dysplasias, strong expression was observed in >90% of the epithelial cells. Four patterns of bcl-2 expression in dysplasias were identified and an increasingly aberrant pattern of bcl-2 expression correlated with an increasing grade of dysplasia (Spearman’s rank correlation, P < 0.0001). Sixty-five per cent of the carcinomas contained bcl-2-positive cells. Patients with non-small-cell lung carcinomas (n = 27) in which >50% of the tumour cells were bcl-2 positive showed a survival advantage compared with those with 0–25% bcl-2-positive cells (P = 0.02). No correlation was found between p53 expression (Walker et al., 1994) and bcl-2 expression in dysplasias or carcinomas.

Keywords: bcl-2; lung cancer; dysplasia; immunohistochemistry

Lung cancer is the commonest cancer in the UK, accounting for one in six of all new cancer cases (Cancer Research Campaign, 1992). Most patients present with already advanced disease, and the prognosis remains poor despite improvements in clinical treatment (Roth, 1992; Southami, 1992; Gazdar, 1994). Conventional screening studies for early detection have had little effect on overall survival (Frost, 1986; Tockman et al., 1992), possibly because lesions that are clinically informative may have already progressed to disseminated disease (Gazdar, 1994).

Lung carcinomas arise after a series of morphological and genetic changes within the bronchial epithelium, and it may take years to progress from normal epithelium to invasive cancer. The morphological changes are thought to progress from hyperplasia, to metaplasia/dysplasia, to carcinoma in situ and finally to invasive and metastatic cancer (Gazdar, 1994; Lee, 1992). Improvements in the treatment of this disease which may prolong survival rely on early recognition of the molecular changes at a time of absent or minimal histopathological change before the acquisition of invasiveness (Gazdar, 1994).

Molecular and cytogenetic changes have been described in preinvasive bronchial lesions (Sundaresan et al., 1992) as well as in normal epithelium adjacent to lung cancers (Lee et al., 1987, 1992; Sozzi et al., 1991). Abnormal expression of the p53 protein has been reported in dysplastic bronchial epithelium and in normal bronchial epithelium of cancer patients (Bennett et al., 1993; Nuorva et al., 1993; Hirano et al., 1994; Walker et al., 1994), suggesting that aberration in p53 function may be a very early process in the development of lung cancers. Such studies prompt further investigations to establish the nature of the changes in dysplastic epithelial cells which may be involved in the development of malignant potential.

The bcl-2 gene codes for a 26 kDa protein with lipophilic character and no substantial homology with any other proto-oncogene products (Clary et al., 1986; Tsujimoto and Croce, 1986; Hockenbery et al., 1990) and may contribute to malignancy by preventing programmed cell death or apoptosis (Hockenbery et al., 1990; Jacobson et al., 1993; Kerr et al., 1993). This proto-oncogene was first described as a result of the chromosomal translocation t(14; 18) seen in a large number of follicular B-cell lines and the majority of malignant human follicular B-cell lymphomas (Tsujimoto et al., 1985; Korsmeyer, 1992). In this translocation the bcl-2 gene on chromosome 18 becomes juxtaposted with the IgH gene on chromosome 14, resulting in overexpression of the bcl-2 protein, and conferring affected lymphocytes with resistance to apoptosis (Cleary et al., 1986; Hockenbery et al., 1990). High levels of bcl-2 expression prevent cell death from a wide variety of cell stresses and cytotoxic chemicals, including growth factor depletion, heat shock, ionising radiation, excess calcium influx and a range of chemotherapeutic drugs (Tsujimoto, 1989; Sentmen et al., 1991; Miyashita and Reid, 1992; Lotem and Sachs, 1993).

In oncogenesis, deregulation of bcl-2 expression may contribute to the accumulation of oncogenic mutations by suppressing the apoptotic deletion of cells that normally follows the induction of DNA damage (Kerr et al., 1993). Pathological expression of bcl-2 has so far been investigated mainly in haematological malignancies (Pezzella et al., 1990; 1991; Korsmeyer, 1992; Piris et al., 1994), but only in a few epithelial or neural tumours (Castle et al., 1993; Leek et al., 1994; Pilotti et al., 1994; Ramini and Lud, 1994; Segal et al., 1994; Silvestrini et al., 1994). The bcl-2 protein is expressed in some small-cell lung cancer (SCLC) cell lines (Ikegaki et al., 1994) and in 28% of non-small-cell lung cancers (NSCLC) (Pezzella et al., 1993). In NSCLC, bcl-2 positivity is associated with better prognosis (Pezzella et al., 1993). Although it is present in a number of lung carcinomas, bcl-2 is absent in differentiated cells of normal bronchial epithelium (Pezzella et al., 1993). Pezzella et al. (1993) suggest that the presence of bcl-2 in differentiated cells may be an indicator of malignancy, but before drawing such conclusions some knowledge of the expression of this molecule in premalignant lesions would be useful.

In this study, we have investigated immunohistochemically the expression of the bcl-2 protein in normal and dysplastic bronchial epithelium and in lung carcinomas. Comparison has been made with the expression of the p53 protein in these tissues, determined previously (Walker et al., 1994).
Materials and methods

Lung tissues

Fifty-six formalin-fixed, paraffin-embedded bronchial biopsies which had been reported to contain dysplastic epithelium were retrieved from the archives at the Histopathology Department, Broadgreen Hospital, Liverpool, UK. In the majority of cases there was a concomitant diagnosis of lung cancer. Dysplasia was graded as mild, moderate or severe as described in Pendleton et al. (1993). Eight formalin-fixed, paraffin-embedded bronchial biopsies and four resection margins, taken from patients who did not have lung cancer at the time of removal and which contained epithelium reported as histologically normal, were also obtained from the files. Thirty-one formalin-fixed, paraffin-embedded specimens of lung carcinoma and their corresponding bronchial resection margins were collected prospectively by Dr N Pendleton from lobectomies or pneumonectomies performed at the Cardiothoracic Centre, Liverpool, UK. Patients received no other form of therapy either before or after surgery and were staged using UICC guidelines. Full clinical data were available for these cases.

Immunohistochemistry

Bcl-2 immunoreactivity was determined using methods similar to those described in Walker (1994), except that microwave antigen retrieval was essential. Sections were microwaved in 10 mM citrate buffer pH 6.0 for 20 min using a 650 W microwave oven at full power before staining. A monoclonal antibody to bcl-2 (clone 124, Dako) was used at 1:40. Negative controls using normal rabbit serum at 1:400 dilution in saline (TBS) in place of the primary antibody and lymphoid tissue as positive control were included in each staining run. Lymphocytes in each section acted as additional internal positive controls.

Sections were reviewed for bcl-2 positivity and the intensity of stained cells scored as negative, weak, moderate or strong. Weak staining was defined as that which was only apparent at high magnification (x 400), while moderate and strong staining was visible at lower magnifications. In normal and dysplastic epithelium, the proportions of bcl-2-positive epithelial cells and their distribution according to the thickness of the epithelium containing these cells was recorded. Dysplasias were classified into four categories (A – D) (Table 1) according to the number and distribution of positive cells and their staining intensity. Inter-observer variability (k) for this classification was 0.87 (95% confidence interval 0.77–0.97). In tumours, the distribution of stained tumour cells across the sections was noted and the percentage of positive cells assessed independently by two pathologists.

Immunoreactivity to p53 in cases that had not previously been investigated was determined as described in Walker et al. (1994) using the CM1 antibody (Novacatra).

Statistical analysis

The significance of associations were determined using the Fisher–Irwin exact probability test or the chi-squared test. Spearman's rank correlation was used to compare the severity of dysplasia with bcl-2 staining patterns. Survival analysis was by the log-rank test. Two-tailed probabilities are quoted for all statistical tests.

Results

Normal epithelium

All bronchial resection margins from lung cancer patients (30/30), bronchial biopsies (8/8) and resected bronchial tissues from non-cancer patients (4/4) showed a similar pattern of immunohistochemical staining in histologically normal epithelium when stained with a monoclonal antibody (clone-124) to the bcl-2 protein. All cases examined showed long stretches of normal epithelium positive for bcl-2, although the intensity of stain varied between cases and within sections; in a few cases areas of epithelium negative for bcl-2 were present. In bcl-2-positive regions of normal epithelium basal cells were stained usually with a moderate to strong intensity, while the more differentiated cells were negative, resulting in a prominent basal staining pattern (Figure 1). The intracellular distribution of this stain was cytoplasmic, with many cells showing perinuclear membranous staining. In some cases occasional brush border cells stained intensely with similar intracellular distribution; sometimes these cells were clearly ciliated. Bronchial glands stained cytoplasmically with variable intensity, some being strongly stained. Perinuclear membranous stain was occasionally noted in glandular epithelial cells. In all tissues scored, lymphocytes were strongly stained.

Dysplastic epithelium

Fifty-six bronchial biopsies with dysplastic epithelium were investigated for expression of the bcl-2 protein. None showed the pattern of staining typical of histologically normal epithelium. All had lost the prominent basal layer of stained cells seen in normal epithelium. In two cases dysplastic epithelium was completely negative for bcl-2, although lymphocytes in the sections were positive. In 28 cases the intensity of the stain in bcl-2-positive cells was weak. In the remaining 26 cases the intensity was similar to or increased compared with bcl-2-positive normal epithelium. In all positive dysplasias the proportion and distribution of positive cells was assessed. In six cases only a few positive very weakly stained cells were evident in basal locations. In 37 cases bcl-2-positive cells of weak or moderate intensity were found in suprabasal locations and in many cases extended throughout the full thickness of the epithelium. In these cases positive cells were present in varying proportions, from focal positivity to up to approximately two-thirds of the epithelial cells. In 11 cases strong expression was observed in the majority of the epithelial cells and throughout the full thickness. In dysplasias all bcl-2-positive cells showed cytoplasmic stain; some showed perinuclear membranous stain. Occasional positive nuclei were observed both in cells in mitosis and in cells not under going mitosis.

Based on the number and distribution of positive cells in the epithelium and their staining intensity, four patterns of bcl-2 expression were identified (Table 1 and Figure 2).

| Table 1 | Expression of the bcl-2 protein in bronchial dysplasia |
|---------|-------------------------------------------------------|
|         | A | B | C | D |
| Intensity of positive cells Distribution of positive cells Proportion of stained cells Number of cases |
| Negative or Weak Basal Up to full thickness Up to full thickness Full thickness |
| <5% | 8 | 22 | 15 | 11 |
Bcl-2 and severity of dysplasia

Dysplasias of all histological grades showed bcl-2 staining patterns A, B and C, while pattern D was only found in severe dysplasia (Table II). Severe dysplasias with the most pleomorphic cells showed the strongest stain. Comparing mild and moderate dysplasias with severe dysplasias, pattern A was more often found in the mild/moderate group ($P = 0.042$), while pattern D was only found in the severe group ($P = 0.004$) (Table II). An increasingly aberrant pattern (from A to D) correlated with an increasing grade of dysplasia (Spearman's rank correlation coefficient of 0.49, $P < 0.0001$). Thus, staining tended to be more prominent, with a greater proportion of bcl-2-positive cells in the upper layers of the epithelium with increasing severity of dysplasia.

In the system of categorisation given in Table I, groups B and C differed only in the staining intensity of bcl-2-positive cells. Even if groups B and C were combined in the Spearman rank correlation analysis a trend towards the more aberrant patterns of bcl-2 expression was still obtained as severity of dysplasia increased (correlation coefficient of 0.46, $P = 0.0002$).

Bcl-2 and p53 expression in dysplastic epithelium

For many of the samples of dysplastic epithelium examined in this study, expression of p53 had already been investigated (Walker et al., 1994). Where sufficient tissue was available, new cases were also examined for p53 expression using the CM1 antibody. There was no correlation between p53 expression and bcl-2 expression.

Bronchial carcinomas

As described above, the expression of the bcl-2 protein was examined in the histologically normal epithelium in bronchial margins from lung carcinoma resections. In the corresponding carcinomas, 20/31 tumours and 16/27 NSCLC's contained bcl-2-positive cells (Table III). Eleven out of 31 tumours were completely negative for bcl-2, even though lymphoid tissues and, in many sections, adjacent normal epithelium stained strongly. A further 14 tumours had bcl-2-negative areas within the tumour. In some bcl-2-positive tumours, staining was focal and patchy, although in positive areas stain was evident in all tumour cells. In others bcl-2 was expressed more intensely at the periphery of tumour islands. Six tumours showed intense stain throughout the entire tumour. In the remaining positive tumours intensity varied between the tumours and within the tumour sections from weak to strong. In tumours stain was cytoplasmic and in some cases perinuclear membranous. In some tumours occasional cells, either in mitosis or with pleomorphic nuclei, showed intense nuclear staining for bcl-2.

Although the number of tumours investigated was small, full clinical information was available for these cases. Bcl-2 expression was found in all histological types (Table III). No correlation was found between the degree of differentiation, the UICC stage or TMN score and bcl-2 expression.

A scatter diagram for the percentage bcl-2-positive tumour cells in the 27 NSCLC's examined in this study is shown in Figure 3. In survival analysis for the NSCLC tumours in this series, the tumour that had 1% tumour cells positive for bcl-2 was included in either the negative group or the positive group with no significant difference to the results obtained. No significant survival advantage was shown for patients with tumours which were negative for bcl-2 compared with those with bcl-2-positive tumours (log-rank test $0.1\%$ vs $20\%$-100%, chi-squared $3.43, P = 0.06$). In contrast, patients in the group whose tumours had $50\%$-100% bcl-2-positive tumour cells had a significantly longer survival than those with $0\%$-25% bcl-2-positive tumour cells (Figure 4) (log-rank test $0.1\%$ vs $50\%$-100%, chi-squared $5.75, P = 0.02$).

| Table II | Bcl-2 expression and severity of dysplasia |
| Grade of dysplasia | A | B | C | D |
|---------------------|---|---|---|---|
| Mild                | 4 | 5 | 2 | 0 |
| Moderate            | 2 | 6 | 2 | 0 |
| Severe              | 2 | 11| 11| 11|

Mild + moderate vs severe: A vs B + C + D, $P = 0.042$* (Fisher's exact test); A + B vs C + D, $P = 0.004$*(chi-squared test); A + B + C vs D, $P = 0.004$*(Fisher's exact test). *Two-tailed probabilities.
Table III Expression of bcl-2 in lung carcinomas

| Histology                        | Any tumour cells positive | Bcl-2 positivity > 20% tumour cells positive | > 50% tumour cells positive |
|----------------------------------|---------------------------|---------------------------------------------|-----------------------------|
| Squamous cell carcinoma          | 8                         | 7                                           | 5                           |
| (n = 14)                         |                           |                                             |                             |
| Adenocarcinomas                  | 6                         | 6                                           | 5                           |
| (n = 11)                         |                           |                                             |                             |
| Large-cell carcinomas            | 1                         | 1                                           | 1                           |
| (n = 1)                          |                           |                                             |                             |
| Small-cell carcinomas            | 1                         | 1                                           | 1                           |
| (n = 1)                          |                           |                                             |                             |
| SCLC/squamous carcinomas         | 1                         | 1                                           | 1                           |
| (n = 1)                          |                           |                                             |                             |
| Adenosquamous carcinomas         | 1                         | 1                                           | 1                           |
| (n = 1)                          |                           |                                             |                             |
| Carcinoids                       | 2                         | 1                                           | 0                           |
| (n = 2)                          |                           |                                             |                             |
| NSCLC                            | 16                        | 15                                          | 12                          |
| (n = 27)                         |                           |                                             |                             |
| Total (n = 31)                   | 20                        | 18                                          | 14                          |

Discussion

Although expression of the bcl-2 protein has so far been investigated in many haematological malignancies and in a number of solid tumours, few studies have examined the distribution of this protein in premalignant lesions.

The bcl-2 protein has been detected by immunohistochemical procedures in a limited number of non-lymphoid tissues under different physiological conditions: in long-lived, post-mitotic cells (neurones), complex organised epithelia (skin and gastric intestinal mucosa) and in glandular epithelium under hormonal control and growth factor control (Hockenbery et al., 1991; McDonnell et al., 1992). In these tissues in which apoptosis accounts for cell turnover, bcl-2 is topographically restricted to the long-lived progenitor cells that renew lineages and selected post-mitotic cells that require an extended lifespan (Hockenbery et al., 1991). In previous studies bronchial epithelial mucosa has been reported either not to express bcl-2 (Hockenbery et al., 1991) or to show positivity in basal cells, with the more differentiated cells being negative (Lu et al., 1993; Pezzella et al., 1993). We have found that cells with their nuclei in the basal layer of histologically normal bronchial epithelium express bcl-2, resulting in a predominantly basal staining pattern. However, although the majority of differentiated cells were negative, in some cases occasional well-differentiated columnar brush border cells also expressed bcl-2. The intracellular distribution of the stain in these occasional differentiated bronchial epithelial cells and the compliance with the predicted pattern of bcl-2 expression in all other cell types in these sections and control lymphoid tissue suggests that these cells do indeed express bcl-2. Furthermore, the monoclonal antibody clone 124 used in this study is reported to have satisfactory specificity and has been used in a number of other studies of both fresh and archival material (Pezzella et al., 1990; Lauwers et al., 1994; Leek et al., 1994; Piolotti et al., 1994; Silvestrini et al., 1994; Ramini and Lu, 1994). These bcl-2-positive differentiated cells were seen in the histologically normal epithelium from cancer and non-cancer patients and are therefore unlikely to reflect an early event in the transformation to malignancy. Glandular epithelium such as breast and thyroid (Hockenbery et al., 1991; Piolotti et al., 1994), and in this study bronchial mucosal glands, express bcl-2, thus bcl-2 positivity in these differentiated bronchial epithelial cells may be related to secretory function.

The bcl-2 protein is an integral membrane protein and is usually associated with a cytoplasmic location, being present in mitochondrial membranes, nuclear outer membranes and endoplasmic reticulum (Korsmeyer, 1992; Akao et al., 1994; Lithgow et al., 1994). In this study, occasional cells with bcl-2-positive nuclei were noted in bronchial dysplasias and carcinomas. In support of this, in some epithelial cell lines the bcl-2 protein has been shown to have occurred transiently in mitotic nuclei (Willingham and Bhalla, 1994; Lu et al., 1994), suggesting that the anti-apoptotic function of bcl-2 extends into mitosis.

Expression of the bcl-2 protein was altered in all bronchial epithelial dysplasias examined, suggesting that deregulation of bcl-2 expression occurs concomitantly with the histological
disorganisation that accompanies dysplasia. It is possible that the loss of the prominently stained basal layer seen in histologically normal epithelium and the appearance of bcl-2-positive cells in the upper layers of the epithelium in dysplastic lesions results from the change from a pseudostratified columnar to a stratified epithelium in which growth control has become aberrant. It is likely that initial changes in bcl-2 expression seen in all dysplasias is secondary to other genetic events which deregulate growth. As bcl-2 is associated in normal cells with protection from apoptosis (Korsmeyer, 1992; Kerr et al., 1994), bcl-2-positive cells in the upper layers of the epithelium may evade stringently controlled normal proliferation and apoptosis and have a growth advantage compared with bcl-2-negative cells. While staining patterns A, B and C were found in all histological grades of dysplasia, pattern D was only observed in some of the severe dysplasias. The change or changes resulting in this pattern of bcl-2 expression may therefore be a relatively late, but not obligatory event in the progression to invasive neoplasia. This expression pattern may result from a specific genetic lesion in either the bcl-2 gene or in a gene which controls bcl-2 expression, occurring relatively late in the development of malignancy. (14; 18) translocations which cause overexpression of bcl-2 in some lymphomas (Korsmeyer, 1992) have not been reported in lung cancers, but immunohistochemical overexpression of bcl-2 has been reported in follicular lymphomas in the absence of this translocation (Pezzella et al., 1990).

Ablerrant expression of the bcl-2 protein has been reported in other premalignant lesions. In gastric epithelial dysplasias, alterations in the spatial distribution of bcl-2-positive cells were observed, with bcl-2-positive cells being present in extended regions of the epithelium (Lauwers et al., 1994). In premalignant keratinocytic tumours, bcl-2 was found to be expressed in 73% of tumours due to Bowen's disease and in 23% of cases of actinic keratosis, in contrast to surrounding bcl-2-negative keratinocytes (Nakagawa et al., 1994).

In the initial design of this study, a limited number of bronchial tumours were examined to determine whether we obtained a similar distribution of bcl-2-positive cells in tumour sections to that reported in the literature for other solid tumours. The bcl-2 protein was found to be distributed similarly but present in a higher percentage of the bronchial carcinomas examined in this study than reported by Pezzella et al. (1993); however, our tumours much lower. Because clinical information was available for these cases, analysis of the clinicopathological data was carried out, although the conclusions drawn are limited by the small number of cases examined. Bcl-2 was expressed in tumours of all grades of histological differentiation. The only positive correlation obtained was for survival, and this only when survival data for patients with tumours with 0–25% bcl-2-positive tumour cells were compared with those for patients with tumours with >50% bcl-2-positive cells. Few studies on the prognostic significance of bcl-2 in NSCLC are as yet published. In the study by Pezzella et al. (1993) a survival advantage for patients with bcl-2-positive tumours was found, particularly for patients with squamous cell carcinomas and in those who were over 60 years of age. In this study the bcl-2-positive group was compared with the bcl-2-negative group (Pezzella et al., 1993). It is notable that Silvestrini et al. (1994), in their study of the prognostic significance of bcl-2 expression in breast carcinomas, compared survival data for patients with more than 30% bcl-2-positive cells with those for patients with lower positivity. The relationship of bcl-2 expression to prognosis and survival in non-haematological malignancies is currently poorly defined. In some studies, bcl-2 expression is associated with a survival advantage (NSCLC, Pezzella et al., 1993; breast, Silvestrini et al., 1994) and markers of good prognosis (breast, Leek et al., 1994), while in others it has no effect (neuroblastoma, Ramini and Lu, 1994) or is related to poor prognosis (neuroblastoma, Castle et al., 1993). While it can be argued that retention of bcl-2 expression in tumours may protect against apoptosis and lead to abnormal accumulation of malignant cells (Korsmeyer, 1992; Kerr et al., 1994), overexpression of bcl-2 has been found to lead to growth inhibition in some cultured cancer cell lines (Pietenpol et al., 1994). These observations suggest that further investigation of bcl-2 expression in a larger series of NSCLCs than hitherto reported would be justified to assess the clinical utility of this marker.

Both the bcl-2 and p53 genes are involved in the genetic control of apoptosis (Haldar et al., 1994; Miyashita et al., 1994a). Recent research has shown that there is a negative response element in the bcl-2 gene through which p53 may directly or indirectly transcriptionally down-regulate the bcl-2 gene (Miyashita et al., 1994b). In breast and some thyroid carcinomas and non-Hodgkin's lymphoma, an inverse correlation between the immunohistochemical expression of p53 and bcl-2 has been demonstrated (Pezzella et al., 1992; Silvestrini et al., 1994; Leek et al., 1994; Pilotti et al., 1994). In contrast, no correlation between p53 and bcl-2 expression was observed in this study in either bronchial dysplasias or carcinomas.

In this study, we have shown that abnormal expression of the bcl-2 protein is present in bronchial dysplasias of all histological grades, suggesting that changes in the expression of this molecule arise early in the transformation of normal to dysplastic epithelium. Further alterations in its expression may occur late in the progression to malignancy. Before expression of this protein could be used as a biomarker for malignancy, further investigation of the expression of this gene and its biological and prognostic significance in lung cancer is essential.

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