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Apoptotic activity is increased in parallel with the metaplasia–dysplasia–carcinoma sequence of the bronchial epithelium

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Summary A high level of apoptotic activity and an independence of apoptosis from the expression of p53 and bcl-2 have been observed in non-small-cell lung carcinoma. We examined 44 samples of normal, metaplastic and premalignant (i.e. mild, moderate and severe dysplasias and carcinoma in situ) bronchial epithelia to evaluate whether differences in the apoptotic activity could already be seen in the stages preceding squamous cell carcinoma of the lung (SQCLC). Apoptotic cells and bodies were visualized by 3′ end labelling. The expression of *p53* and members of the *bcl-2* gene family, such as *bcl-2*, *bax* and *mcl-1*, were determined immunohistochemically with specific antibodies. The relative number of apoptotic cells and bodies [apoptotic index (AI%)] was already increased threefold as the normal bronchial epithelium changed to squamous metaplasia, and the AIs of the dysplastic lesions were about four times higher than those of the normal epithelium. Apoptosis was significantly associated with cell proliferation, as determined by proliferating cell nuclear antigen (PCNA) immunohistochemistry. However, the extent of apoptosis did not correlate with the expression of p53, bcl-2, bax and mcl-1. We conclude that, in the metaplasia–dysplasia–carcinoma sequence in the lung, the elevation of the AI% is an early event associated with cell proliferation activity, but is independent of the expression of p53, bcl-2, mcl-1 and bax.

Keywords: apoptosis; cell proliferation; p53; bcl-2-family; bronchial dysplasia

Squamous cell carcinoma of the lung (SQCLC) is generally associated with smoking and is preceded by clinically and histologically definable preinvasive lesions of the bronchial epithelium (Auerbach et al, 1957, 1962, 1979). Squamous cell metaplasia, developing from basal cell hyperplasia, is the earliest recognizable morphological, but not yet neoplastic, change. Mild, moderate and severe dysplasias and squamous cell carcinoma in situ are already premalignant, but still non-invasive lesions (World Health Organization, 1981). The malignant phenotype of invasive carcinoma is believed to develop as a result of aberrations in the expression and function of oncogenes, proto-oncogenes and tumour-suppressor genes (e.g. *bcl-2* and *p53*), leading to a growth advantage of the neoplastic cells. *p53* and the genes of the *bcl-2* family, such as *bcl-2*, *bax* and *mcl-1*, are regulatory genes of apoptosis, an event in which single cells die following a highly defined, predetermined programme. During this event, several morphological changes lead to the formation of membrane-bound apoptotic bodies, which are then phagocytosed by neighbouring cells (Kerr et al, 1972).

Mutations in the *p53* tumour-suppressor gene are found in 37% of all human malignancies and in 60% of lung carcinomas (Greenblatt et al, 1994). Aberrant expression of p53 has also been detected in preinvasive lesions of the bronchial epithelium (Sozzi et al, 1992; Sundaresan et al, 1992; Vähäkangas et al, 1992; Bennett et al, 1993; Nuorva et al, 1993; Hirano et al, 1994; Walker et al, 1994), in which it is associated with the severity of dysplasia (Sundaresan et al, 1992; Bennett et al, 1993; Nuorva et al, 1993; Hirano et al, 1994; Walker et al, 1994).

The effects of the ever-expanding *bcl-2* gene family on apoptosis are under intensive research. The first member of this group was the *bcl-2* proto-oncogene, which was first identified in follicular lymphomas (Tsujimoto et al, 1984; Bakhshi et al, 1985) and was later found to be an inhibitor of apoptosis induced by a wide variety of stimuli (Reed, 1994). In normal skin, bcl-2 is expressed in the basal epithelial cells (Hockenbery et al, 1991; Lu et al, 1993). In bronchial dysplasias, bcl-2 expression can be detected throughout the epithelial layer, and the irregularity of the staining pattern increases concurrently with the degree of dysplasia (Walker et al, 1995). *bax*, a member of the *bcl-2* gene family, produces a protein with approximately 21% amino acid sequence homology with *bcl-2* (Oltvai et al, 1993). When overexpressed, bax forms homodimers and a cell death-promoting signal is accelerated. In contrast, when bcl-2 is in excess, it heterodimerizes with bax and cell death is repressed (Oltvai et al, 1993). Thus, it has been suggested that the ratio of bcl-2 to bax determines the susceptibility of a cell to apoptosis (Oltvai et al, 1993). Mcl-1, which displays functional similarity to bcl-2, is capable of binding to bax and suppressing bax-induced cytotoxicity (Bodrug et al, 1995).

Recently, it was shown that members of the *bcl-2* family regulate apoptosis through their ability to alter the mitochondrial membrane potential and to form ion channels (Kroemer, 1997;

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Minn et al, 1997; Yang et al, 1997). This leads to the liberation of caspase-activating substances, such as cytochrome c (CK) and apoptosis-inducing factor (AIF), from the mitochondria into the cytosol causing apoptosis of the cell. The proapoptotic bax has been shown to induce the liberation of such caspase-activating substances, whereas the antiapoptotic bcl-2 and bcl-x_L inhibit it (Kroemer, 1997; Minn et al, 1997; Yang et al, 1997).

A high level of apoptotic activity in tumours is generally considered to signify slower tumour growth and a better prognosis. In our previous work, however, enhanced apoptosis in non-small-cell lung carcinoma (NSCLC) was associated with shortened survival (Törmänen et al, 1995). In addition, the relative number of apoptotic cells and bodies was independent of the expression of p53 and bcl-2 (Törmänen et al, 1995). In human colorectal tubular adenomas, apoptotic cells are seen more frequently in high- than in low-grade dysplasias (Arai and Kino, 1995). In the light of these observations, it was of interest to examine whether there is a trend towards increased apoptosis in the premalignant lesions preceding SQCLC and whether aberrations in the expression of apoptosis-regulating genes, such as *p53*, *bcl-2*, *bax* and *mcl-1*, can already be seen at this stage. We analysed the extent of apoptosis in 44 bronchial tissue samples representing normal bronchial epithelium, squamous metaplasia, bronchial epithelial dysplasia and carcinoma in situ.

MATERIALS AND METHODS

Case selection and classification of lesions

Forty-four formalin-fixed samples containing premalignant, non-invasive, bronchial lesions were collected from the archives of the Departments of Pathology, Oulu University Hospital, Finland, and Central Finland Health Care District. All specimens were histopathologically re-evaluated according to the World Health Organization Histological Typing of Lung Tumours (1981), and representative tissue samples were chosen for further studies. The bronchial epithelium was normal in 14 cases, squamous metaplasia was found in eight cases, mild epithelial dysplasia in three, moderate dysplasia in eight and severe dysplasia in six cases. The lesions in five samples were graded as squamous cell carcinoma in situ.

3′ End labelling of apoptotic DNA fragments

Apoptotic cells and bodies in tissue sections were detected using an ApopTag in situ Apoptosis Detection Kit (Oncor, Gaithersburg, MD, USA). The instructions laid out by the manufacturer were followed with a few modifications, as described previously (Törmänen et al, 1995). A cell was defined as apoptotic if the whole nuclear area was positively labelled, whereas apoptotic bodies were identified as small, positively labelled, globular fragments. All positively labelled cells and bodies fulfilled the morphological criteria for apoptosis as described by Kerr et al (1972), i.e. condensation of the nucleus, cell shrinkage, cytoplasmic budding to form membrane-bound fragments, and detachment from surrounding cells.

The number of apoptotic cells and bodies in the epithelial tissue was counted in ten high-power fields (HPFs; objective ×40, field diameter 400 μm) when possible. In some specimens, the amount of epithelium was not sufficient; in these cases, a minimum of four HPFs were studied. Apoptotic bodies likely to be originating from the same apoptotic cell were recorded as one apoptotic body. The quantity of apoptotic cells and bodies, or apoptotic index (AI%), is expressed as a percentage of the whole epithelial cell population.

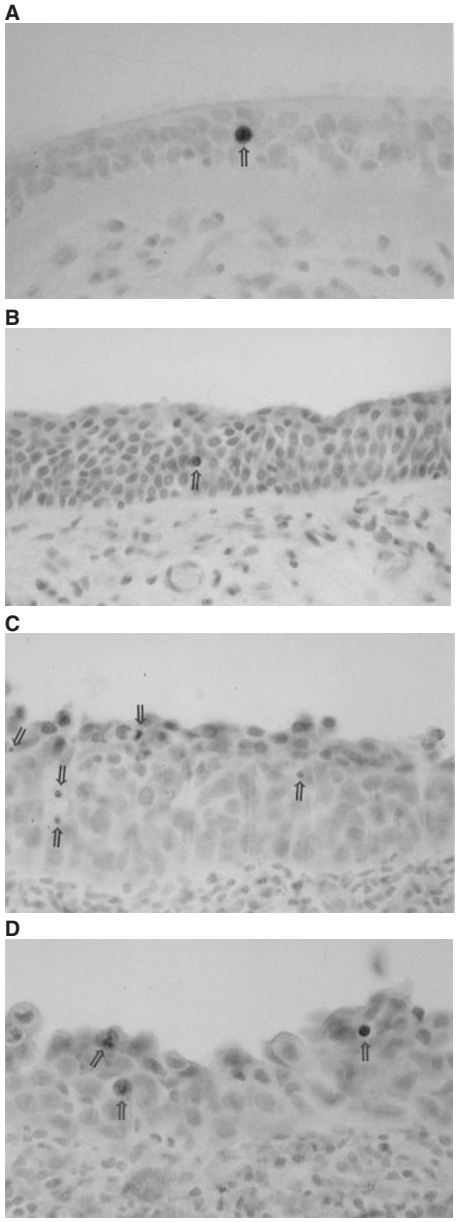

Figure 1 The extent of apoptosis in normal bronchial epithelium and in the metaplasia–dysplasia–carcinoma sequence of the bronchus. 3′ End labelling of apoptotic DNA. (**A**) One apoptotic cell (arrow) in normal bronchial epithelium. (**B**) One apoptotic cell (arrow) in metaplastic epithelium. (**C**) Four apoptotic bodies (arrows) in a moderately dysplastic area of the epithelium. (**D**)Three apoptotic cells (arrows) in a squamous cell carcinoma in situ

Immunohistochemistry for p53

The immunohistochemistry for p53 was performed as described previously by Törmänen et al (1995), using a polyclonal antibody CM-1 (Novocastra Laboratories, Newcastle upon Tyne, UK) at a dilution of 1:1000.

Table 1 The extent of apoptosis in normal and metaplastic bronchial epithelia, bronchial dysplasias and squamous cell carcinoma in situ of the lung

	Apoptotic cells (%) mean (range)	Apoptotic bodies (%) mean (range)	Apoptotic index (%) mean (range)	Number of cases studied
Normal epithelium	0.18 (0.00–0.39)	0.16 (0.00–0.45)	0.34 (0.00–0.69)	14
Squamous metaplasia	0.34 (0.00–0.88)	0.59 (0.00–1.00)	0.92 (0.40–1.57)	8
Mild dysplasia	0.66 (0.27–1.11)	0.79 (0.41–1.13)	1.45 (0.68–1.94)	3
Moderate dysplasia	0.47 (0.00–1.11)	1.19 (0.09–3.60)	1.66 (0.18–4.40)	8
Severe dysplasia	0.64 (0.32–1.29)	0.73 (0.20–1.34)	1.37 (0.52–2.63)	6
Carcinoma in situ	0.51 (0.21–0.83)	0.98 (0.21–2.48)	1.49 (0.63–3.26)	5

Apoptotic indices were significantly higher in preinvasive bronchial lesions than in normal and metaplastic epithelia ($P < 0.001$).

Table 2 Distribution of p53 positivity in normal, metaplastic and dysplastic bronchial epithelia

	Immunoreactivity for p53							
Type of epithelium	Negative	<1%	1–5%	6–10%	11–40%	>40%	NA	Total
Normal	14							14
Squamous cell metaplasia	7	1						8
Mild dysplasia	3							3
Moderate dysplasia	6			1	1			8
Severe dysplasia	4		2					6
Carcinoma in situ				2	1	1	1	5
Total number of sections	34	1	2	3	2	1	1	44

Abnormal accumulation of p53 protein (>1% of p53-positive nuclei) was more often observed in mild, moderate and severe dysplasias and carcinoma in situ than in areas of normal and metaplastic epithelia ($P < 0.001$).

The cases were divided into six groups according to the percentage of p53-positive nuclei in the whole epithelial layer as follows: 0, negative; (+), <1% of positive nuclei; +, 1–5%; ++, 6–10%; +++, 11–40%; and ++++, >40% of positive nuclei.

Immunohistochemistry for proliferating cell nuclear antigen (PCNA), bcl-2, bax and mcl-1

The procedure of bcl-2 immunohistochemistry using anti-human bcl-2 antibody (clone 124, Dako, Glostrup, Denmark) is described in our previous report (Törmänen et al, 1995). The immunohistochemistry for PCNA, bax and mcl-1 was performed similarly using the following antibodies: monoclonal anti-PCNA 19A2 (BioGenex, San Ramon, CA, USA) at a dilution of 1:20, incubated 30 min at room temperature; polyclonal anti-human bax (Pharmingen, San Diego, CA, USA) at a dilution of 1:1000, incubating overnight at room temperature; polyclonal anti-human mcl-1 (Pharmingen) at a dilution of 1:1000, incubating overnight at room temperature.

The positivity of bcl-2 was evaluated separately for the basal epithelial layer and the other layers, and three groups were formed based on the following classification: 0, negative; 1, positive only in basal cell layer; and 2, positive in suprabasal epithelium. The proliferative activity is defined as the percentage of PCNA-positive nuclei in the whole epithelial cell population. To describe the expression of bax and mcl-1 in the bronchial epithelium, the intensity of the immunostaining was evaluated as follows: 1, weak cytoplasmic staining; 2, moderate cytoplasmic staining; 3, strong cytoplasmic staining. Based on the quantity of the staining, the specimens were divided into three groups: 1, 1–25% of positive cells; 2, 26–50% of positive cells; 3, >50% of positive cells, for bax or mcl-1. A combined index based on both the intensity and the quantity of the immunostaining was determined by adding the qualitative and the quantitative scores and, based on this, two groups were formed: weak immunoreactivity (scores 0–4) and strong immunoreactivity (scores 4–6).

Control stainings

A SQCLC previously shown to be p53 positive (Soini et al, 1992) was used as a positive control for p53 immunostaining. A hyperplastic lymph node was used as a control for the labelling of apoptotic DNA fragments and for bcl-2, PCNA, bax and mcl-1 immunohistochemistry. Negative controls for all immunostainings were obtained by substituting the primary antibody with PBS.

Statistical analysis

The statistical analysis was performed with the SPSS for Windows program package (Chicago, IL, USA). The values of the apoptotic indices are reported as means with ranges. The significance of the associations was determined by using the χ^2 test, Fisher's exact probability test, or Student's two-tailed *t*-test. Probability values of less than 0.05 were considered to be significant.

Table 3 PCNA immunoreactivity

	Percentage of PCNA-positive nuclei		
Type of epithelium	**Mean**	**Range**	**Total number of sections**
Normal	1	0–5	14
Squamous cell metaplasia	6	1–15	8
Mild dysplasia	24	8–40	3
Moderate dysplasia	10	5–20	8
Severe dysplasia	23	16–28	6
Carcinoma in situ	28	8–40	5

Table 4 p53 and PCNA immunoreactivity

	PCNA immunoreactivity, number of cases	
p53 status	**≤5% Positive nuclei**	**>5% Positive nuclei**
Negative	17	9
Positive	0	6

p53-positive nuclei were more frequently detected in areas in which over 5% of the nuclei were positive for PCNA than in areas of lower proliferation rate ($P = 0.006$).

RESULTS

Apoptosis in normal bronchial epithelium and premalignant lesions

Bronchial epithelial cells in different stages of apoptosis could be easily detected after 3′ end labelling. Apoptotic cells, as well as apoptotic bodies comprising small membrane-bound fragments representing the last phase of apoptosis, were distributed throughout the whole epithelial layer in all types of epithelium studied (Figure 1A–D). The extent of apoptosis is presented in Table 1. Interestingly, the mean apoptotic indices of the metaplastic epithelium were about threefold higher than those of the normal bronchial epithelium. In mild and moderate dysplasias, the apoptotic indices were over four times higher than normal epithelium. When the samples were divided into two groups, the apoptotic indices were significantly higher in premalignant lesions (i.e. mild, moderate and severe dysplasias and carcinoma in situ) than in normal and metaplastic bronchial epithelia ($P = 0.001$; Table 1).

Immunoreactivity of p53 and PCNA in normal bronchial epithelium and preinvasive lesions

The distribution of p53 positivity is presented in Table 2. No positivity could be seen in normal epithelium. In dysplasias, p53-positive nuclei were mainly seen as groups throughout the epithelial thickness. Abnormal accumulation of p53 protein (>1% of p53-positive nuclei) was more often observed in mild, moderate and severe dysplasias and in carcinoma in situ than in areas of normal and metaplastic epithelia ($P = 0.009$; Table 2).

The extent of cell proliferation, as measured by PCNA immunohistochemistry, is shown in Table 3. PCNA-positive nuclei were significantly more abundant in premalignant lesions than in normal and metaplastic bronchial epithelia ($P < 0.0001$). p53-positive nuclei were more frequently detected in areas in which over 5% of the nuclei were positive for PCNA than in areas presenting lower proliferation rates ($P = 0.006$; Table 4).

Expression of bcl-2, bax and mcl-1

A uniform cytoplasmic staining pattern was seen in bcl-2-positive cells. In 13 samples of normal epithelium, bcl-2 positivity was detected in the basal cell layer. In two cases of squamous cell metaplasia, a positive reaction could be observed either in the basal cell layer or in the suprabasal layers of the epithelium. bcl-2 positivity was seen throughout the whole epithelial thickness in 7 out of 21 cases of premalignant epithelial lesions, and only in the basal cell layer in seven cases. Fourteen cases were negative for bcl-2. Data could not be obtained in one case because of depletion of tissue from the paraffin block.

Of the 18 cases showing strong immunoreactivity for bax (with a combined score, based on both the intensity and the quantity of the staining, higher than 4), ten were samples of normal or metaplastic bronchial epithelia, five were low-grade dysplasias (i.e. mild or moderate) and three were high-grade dysplasias (i.e. severe dysplasia or carcinoma in situ). Seventeen cases (eight normal or metaplastic, four low-grade and five high-grade dysplasias) showed strong immunoreactivity for mcl-1. No differences were found between the normal–metaplastic group and the premalignant group in either bax or mcl-1 immunoreactivities. Similarly, no correlation was found between the expression of bcl-2 and bax when all the samples were evaluated as a single group or in smaller groups.

Apoptosis in relation to p53, PCNA, bcl-2, bax and mcl-1

The mean apoptotic indices of all the bronchial epithelial lesions studied were higher in p53-positive than in p53-negative lesions ($P = 0.009$; Table 5). However, no correlation between the extent of apoptosis and positive p53 immunostaining was found when the premalignant groups were evaluated separately (Table 5). A high level of proliferative activity, as measured by PCNA immunohistochemistry, was associated with a higher apoptotic index ($P = 0.012$). No correlation was found between apoptotic activity and the expression of bcl-2, bax and mcl-1 (Table 6).

Table 5 Apoptotic indices and p53 expression

Type of epithelium	p53 status	Apoptotic index (%)		
		Mean	Range	Number of sections
Normal	p53 –	0.34	0.00–0.69	14
	p53 +			0
Squamous cell metaplasia	p53 –	0.91	0.40–1.57	7
	p53 +	1.00		1
Mild dysplasia	p53 –	1.45	0.68–1.94	3
	p53 +			0
Moderate dysplasia	p53 –	1.45	0.18–4.40	6
	p53 +	2.30	2.00–2.59	2
Severe dysplasia	p53 –	1.13	0.52–1.62	4
	p53 +	1.84	1.04–2.63	2
Carcinoma in situ	p53 –			0
	p53 +	1.49	0.63–3.26	5

Table 6 Apoptotic activity and the expression of bcl-2, mcl-1 and bax. Figures represent number of cases

	Apoptotic activity				Total number of cases
	Normal and metaplastic tissue		Premalignant tissue		
	≤0.74%	>0.74%	≤0.74%	>0.74%	
bcl-2					
Negative	4	3	0	7	14
Positive	13	2	4	10	29
mcl-l					
Index ≤4	2	1	0	5	8
Index >4	8	0	3	6	17
bax					
Index ≤4	3	0	1	6	10
Index>4	9	1	1	7	18

DISCUSSION

Apoptosis is one of the most actively studied phenomena in the field of cell biology at the moment. There are several reports describing its extent in neoplasias of different tissues, and most indicate that apoptosis is more frequent in malignant than in normal tissue (Lipponen and Aaltomaa, 1994; Aihara et al, 1995; Arai and Kino, 1995; Bardeesy et al, 1995; Staunton and Gaffney, 1995; Törmänen et al, 1995; Soini et al, 1996). However, studies of preneoplastic lesions are rare and, to our knowledge, none have been published on bronchial dysplasias. The main purpose of the present work was to study the extent of apoptosis in normal and metaplastic bronchial epithelia and in non-invasive, premalignant lesions preceding SQCLC, i.e. mild, moderate and severe dysplasias and squamous cell carcinoma in situ.

We found the relative number of apoptotic cells and bodies (apoptotic index) to increase as the normal epithelium of the bronchus gradually alters to a premalignant lesion. In normal epithelium, single cells undergoing apoptosis were seldom seen. We assume that, in the normal epithelium, the apoptotic programme functions normally, eliminating cells with a genetic defect. Hence, an increased number of apoptotic cells and bodies in metaplastic and dysplastic lesions might be the result of normal activity, rather than of an impaired expression of apoptosis-regulating genes, such as *p53*, thus reflecting a larger number of damaged cells in the lesion.

In line with some previous studies, we found aberrant p53 expression to be associated with the severity of the dysplasia (Sundaresan et al, 1992; Bennett et al, 1993; Nuorva et al, 1993; Hirano et al, 1994; Walker et al, 1994). We also found the p53 protein to accumulate in lesions preceding SQCLC, starting with mild dysplasia. The apoptosis-inducing function of *p53* has been thought to vanish whenever mutations occur (Lowe et al, 1994; Bardeesy et al, 1995). This hypothesis is supported by the results from Wilms' tumours (Bardeesy et al, 1995) and small-cell lung carcinomas (Eerola et al, 1997), in which there was an inverse correlation between the extent of apoptosis and *p53* mutations or p53 immunoreactivity. In contrast, Lipponen and Aaltomaa (1994) demonstrated that p53-positive bladder tumours show a significantly higher number of apoptotic cells and bodies than p53-negative tumours. In non-small-cell lung carcinomas, we found no

correlation between these two parameters (Törmänen et al, 1995). In this study, both the apoptotic index and the degree of p53 protein accumulation increased with the severity of the dysplasia. These strikingly different results on the relationship between p53 expression and apoptosis in different types of carcinoma emphasize the need to study the effects of different mutations on the apoptosis-regulating characteristics of the *p53* gene. The study of the expression of other apoptosis-regulating genes in different tissues would also be of value because the lost ability of *p53* to induce apoptosis might be compensated by other, more powerful regulators. One might also hypothesize that the elevation of the apoptotic indices in bronchial dysplasias is due to a normal, apoptosis-promoting function of *p53*. In our opinion, this theory is contradicted by the observation that the accumulation of immunohistochemically detectable p53 protein increased in parallel with the severity of the dysplasia. The p53 protein in the lesions would thus be mutated, or otherwise inactivated, and unable to induce apoptosis.

The results of this study indicate that both the rate of cell proliferation and the quantity of cell death are altered in parallel with the morphological changes. We found a direct correlation between the extent of apoptosis and the rate of cell proliferation, as evaluated by PCNA immunohistochemistry. A similar association has been previously shown in colorectal tubular and villous adenomas (Arai and Kino, 1995) and carcinomas (Baretton et al, 1996), as well as in gastric (Koshida et al, 1997), endometrial (Saegusa et al, 1996), breast (Lipponen et al, 1994) and bladder (King et al, 1996) carcinomas.

The expression of bcl-2 in this selection of normal bronchial epithelium and dysplasias of different severity was quite similar to that reported by Walker et al (1995). In our work, however, no increase of bcl-2 expression could be seen as the severity of dysplasia increased. Previously, we found no correlation between the extent of apoptosis and the expression of bcl-2 in non-small-cell lung carcinomas (Törmänen et al, 1995). Similarly, no association between these two factors could be found in bronchial dysplasias. Furthermore, no correlation was found between apoptosis and the expression of bax and mcl-1.

The independence of apoptosis from the expression of p53 and the bcl-2 family proteins suggests that the apoptotic signalling pathway in bronchial dysplasias functions independently of these factors. As it has been shown that the *Fas* receptor is expressed in lung tumours (Hellquist et al, 1997), the main pathway leading to apoptosis in bronchial dysplasias preceding SQCLC could also include the activation of the *Fas* receptor. In this system, the *Fas* ligand, bound for example to the cytotoxic lymphocytes, binds to the oligomerized *Fas* receptor which then, via FADD-MORT, activates FLICE (i.e. caspase 8) and, thus, the caspase cascade, leading to apoptosis without the involvement of *p53* and the bcl-2-related proteins (Muzio et al, 1996; Nagata, 1997). This could explain the independence of apoptosis from both the expression of p53 and of the gene products of the *bcl-2* family in bronchial dysplasias and NSCLC.

Based on these results, it seems that the elevation of the apoptotic index is an early event in the process in which the normal bronchial epithelium changes to squamous cell carcinoma in situ, and that the increase in the apoptotic activity is associated with the severity of the bronchial premalignant lesion, i.e. dysplasia. The highest apoptotic indices were found in severe dysplasias and carcinoma in situ, exceeding even those reported for SQCLC in our previous study (Törmänen et al, 1995). It is possible that the high apoptotic activity in a premalignant lesion reflects an attempt to eliminate genetically damaged cells and that, in the following invasive carcinoma, the aggregated mutations somehow interfere with the apoptosis-regulating mechanisms. In line with our observations, Birchall et al (1995) and Ishida et al (1996) have demonstrated that the apoptotic activity is higher in premalignant gastric lesions and in dysplasias of the oral cavity than in the corresponding invasive carcinomas.

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