Enhanced Apoptosis Predicts Shortened Survival in Non-Small Cell Lung Carcinoma

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ABSTRACT

This study was undertaken to determine the extent of apoptosis in lung carcinoma and to evaluate it as a prognostic marker. A series of 75 lung carcinomas (47 squamous cell carcinomas, 24 adenocarcinomas, 3 small cell carcinomas, and 1 large cell carcinoma) was evaluated for the extent of apoptosis by using the 3' end-labeling method of DNA in tissue sections. Apoptosis was correlated with the rate of cell proliferation, the immunohistochemically detectable p53 and bcl-2, the extent of tumor necrosis, and the survival data. The end-labeling method allowed a precise evaluation of the extent of apoptosis. In tumor tissue, the number of apoptotic bodies was roughly 2-fold greater than the number of apoptotic cells, whereas in nonneoplastic control tissues, the ratio was 1:1. The apoptotic indexes (percentages of apoptotic cells and bodies among tumor cells) were slightly higher in adenocarcinoma than in squamous cell carcinoma. There was no association between the extent of apoptosis and the expression of proliferating cell nuclear antigen or p53. On the other hand, tumor necrosis correlated significantly with proliferating cell nuclear antigen and p53 positivity (P = 0.00025 and 0.00087, respectively). Surprisingly, the extent of apoptosis was also found to be independent of the expression of bcl-2. Patients with apoptotic indexes greater than 1.5% had significantly shorter survival time than patients with apoptotic indexes equal to 1.50% or less (P < 0.01 by log rank). A bcl-2 positive tumor had a poor prognosis (P < 0.002 by log rank). By multivariate analysis, enhanced apoptosis showed a 1.9-fold risk (P = 0.04), and p53 positivity showed a 2.3-fold risk (P = 0.005) for a shortened survival. We conclude that both enhanced apoptosis and p53 positivity are independent prognostic markers in non-small cell lung carcinoma, predicting shortened survival time of the patients.

INTRODUCTION

The rate of tumor growth is dependent on both the proliferative activity and death rate of the tumor cells. Traditionally, proliferative activity, as measured, e.g., by mitotic counts, has been considered the principal determinant of the growth rate and an important prognostic factor. The role of cell death, on the other hand, is less well defined, mainly due to difficulties in evaluating it in quantitative terms. There are two major pathways that lead to cell death. One is necrosis, a process in which tumor cells die due to some extrinsic factors, such as starvation. Another is apoptosis, a regulated process in which the cells die due to an intrinsic mechanism. Although necrosis is a poorly defined phenomenon without any predetermined machinery, apoptosis seems to be a highly programmed event with successive biochemically and morphologically defined stages. Due to a recent progress in the understanding of its molecular mechanisms that involve, e.g., identification of its regulators and effectors, apoptosis has become amenable to quantitative scrutiny more on par with our current techniques to quantitate proliferative activity of tumor cells.

Two of the best known examples of genes regulating apoptosis are bcl-2 and p53. bcl-2 encodes for a Mr 25,000 protein localized in the mitochondrial membrane, nuclear envelope, and endoplasmic reticulum (1). It is known to inhibit apoptosis induced by a wide variety of stimuli (2). In follicular lymphomas, the bcl-2 gene is translocated to a vicinity of an active promoter, which leads to an increased level of bcl-2 protein expression, with a resultant neoplastic transformation (3, 4).

p53 is a tumor suppressor gene that is mutated in 37% of all human malignancies (5). The tumor suppressor function of wild-type p53 is based on its capacity to produce G1 arrest and/or to direct cells toward apoptosis (6–9). The apoptosis-promoting capacity is presumably due to the ability of p53 to transactivate bac, a gene that encodes an inhibitor of bcl-2 protein (10). When in excess, bac protein inhibits the function of bcl-2, which then leads to increased apoptosis. The loss of normal p53 function, e.g., by incapacitating mutations, on the other hand, delays apoptosis, as seen, for instance, in irradiating human lymphoblasts defective in p53 function (11). Thus, mutated p53, which due to its extended half-life can be detected by immunohistochemical techniques, could be expected to be found in tumors and be associated with impaired apoptosis.

There are several studies that suggest that p53 and bcl-2 play an important role in the pathogenesis and progression of lung carcinoma. For instance, 60% of lung tumors carry a p53 mutation (5), and overexpression of p53 protein has been associated with poor prognosis in lung carcinoma (12–15). Expression of bcl-2 protein, on the other hand, has been found in 25% of squamous cell carcinomas and in 12% of adenocarcinomas of the lung, and it seems to predict a better prognosis (16). There are, however, no studies on the extent of apoptosis in lung carcinoma and its effect on prognosis.

In this study, we analyzed 75 cases of lung carcinoma for the rate of proliferation and for the extent of apoptosis. The proliferation rate was determined immunohistochemically by using an antibody to PCNA. The extent of apoptosis was determined by detecting and counting apoptotic cells by in situ 3' end labeling of the DNA. The level of p53 and bcl-2 expression was determined immunohistochemically to allow for assessment of their role in the regulation of proliferation and destruction of the tumor cells. Also, the extent of necrosis was evaluated and correlated with the other parameters. Finally, these parameters were correlated with the survival data of the patients to allow for evaluation of their prospective use as prognostic markers in lung carcinoma.

MATERIALS AND METHODS

Tumor Material. Lung carcinomas of 75 patients, 70 men (93.3%) and 5 women (6.7%), ages 48–80 years (mean, 62 years), from the Oulu University Central Hospital during the years 1978–1980 were included in this study. Pulmonary lobes were fixed intrabronchially with 10% buffered formalin for 24 h. Representative tissue samples were then collected, embedded in paraffin, and stained with hematoxylin and eosin and periodic acid-Schiff for routine histology. All tumors were evaluated for histopathological diagnosis and

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3 The abbreviations used are: PCNA, proliferating cell nuclear antigen; HPF, high-power field; CI, con-
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classified according to the WHO International Histological Typing of Lung Tumors (17). Forty-seven of the patients had squamous cell carcinoma, 24 adenocarcinoma, 3 small-cell carcinoma, and 1 large cell carcinoma. The postsurgical TNM status of each non-small cell carcinoma was reevaluated considering histopathological analysis of surgical specimens; 33.8% (n = 24) were T1N0, and 45.1% (n = 32) were T2N0, whereas nodal metastases were found in 8.4% (n = 6) of the tumors classified as T1 or T2. All remaining carcinomas (n = 9) were T3N0 to T4N0. Representative sections were chosen for the labeling of apoptotic cells and for immunostainings. The relative volume of necrosis in each tumor was also determined. The clinical follow-up data of the patients were collected from the hospital records. Follow-up times varied from 1 to 161 months (mean, 54 months). Nineteen (25.3%) of the patients were alive, and 52 (69.3%) had died of lung carcinoma.

3' End Labeling of DNA in Apoptotic Cells. The 3' end labeling of apoptotic cell DNA was performed by using an ApopTag in situ apoptosis detection kit (Oncor, Gaithersburg, MD) following the instructions laid out by the manufacturer, with a few modifications. Briefly, after dewaxing and rehydration, the sections were incubated with 20 µg/ml proteinase K (Boehringer Mannheim GmbH, Mannheim, Germany) at room temperature for 15 min. Endogenous peroxidase activity was quenched in 2% hydrogen peroxide in PBS (pH 7.2). Terminal transferase enzyme was used to catalyze the addition of digoxigenin-labeled nucleotides to the 3'-OH ends of the fragmented DNA. Subsequently, antidigoxigenin-peroxidase solution was applied on the slides. Diaminobenzidine-hydrogen peroxide was used to develop the color reaction. Specimens were counterstained lightly with hematoxylin. A part of the tumor material was also labeled with the in situ apoptosis detection kit (Oncor) using a fluorescein-labeled antidigoxigenin antibody instead of the peroxidase-labeled antibody.

Determination of Apoptotic Indexes in Lung Carcinoma and Control Materials. Two different types of indexes for apoptosis were determined. First, the absolute extent of apoptosis/HFP was determined by counting the number of apoptotic cells and bodies in a minimum of 10 HFPs (objective, ×40; diameter of the field, 400 µm) and dividing it by the number of fields. This was done separately for apoptotic cells and bodies. Apoptotic bodies occurring in distinct groups, and likely to be originating from one and the same apoptotic cell, were recorded as one apoptotic body. Second, the relative number of apoptotic cells and bodies in the entire tumor cell population was determined by counting their numbers in 10 HFPs. The index was given as a percentage of viable tumor cells.

Controls for Apoptosis. As a positive control for apoptosis, we used peripheral blood lymphocytes treated with dexamethazone. Lymphocytes were separated from heparinized blood using lymphocyte separation medium (Ficoll-Paque; Pharmacia LKB Biotechnology Inc., Uppsala, Sweden). After 48 h culture, lymphocytes were treated for 24 h either with 1 µM, 5 µM, or 10 µM dexamethazone or without it. The cells were then harvested, embedded in 2% agar, and, after solidification, further embedded in paraffin. In a few experiments, the lymphocytes were fixed in 4% glutaraldehyde, immersed in 2% agar, and, after solidification of the agar, embedded in plastic medium for electron microscopy.

For control purposes, we also evaluated the extent of apoptosis in the adjoining uninjured areas and in the resection margins of the tissue blocks containing the tumors. This was done separately for bronchial epithelium, serous and mucous glands in the bronchial wall, alveolar macrophages, and interstitial cells of lung parenchyma without specifying the cell types. In hyperplastic lymph nodes, germinal centers and extralacular areas, but not sinuses, were also evaluated for apoptotic activity.

Immunohistochemical Stainings. Sections of 4 µm in thickness were cut and adhered to glass slides pretreated with poly-L-lysine (Sigma Chemical Co., St. Louis, MO) and air dried overnight. The sections were then dewaxed in xylene and rehydrated in graded alcohol. The endogenous peroxidase activity was blocked by 0.1% hydrogen peroxide, and nonspecific binding was blocked by 20% FCS.

Immunohistochemistry for PCNA and bcl-2. To detect PCNA protein expression in the proliferating cells, we used monoclonal antibody PC10 (Dako, Glostrup, Denmark). A monoclonal antibody (clone 124) directed against bcl-2 oncogene protein was also obtained from Dako. Prior to the 30-min incubation with these primary antibodies (both diluted 1:50), the sections were heated in 10 mm citric acid monohydrate (pH 6.0) in a microwave oven for 3 min. Following a 30-min incubation with a biotinylated secondary antiserum antibody (Dakopatts, Copenhagen, Denmark), diluted 1:300, the immunostaining was performed with the avidin-biotin-peroxidase complex technique (18) using diaminobenzidine-hydrogen peroxide as a chromogen. The sections were counterstained lightly with hematoxylin. A tissue section of hyperplastic lymph node was used as a positive control for both PCNA and bcl-2.

The proportion of PCNA-positive cells was counted in at least six HPFs (objective, ×40; diameter of the field, 400 µm). PCNA positivity was classified as follows: group 1, less than 1% of the nuclei positive; group 2, 1—10%; group 3, 11—50%; group 4, 51—75%; and group 5, >75%. A tumor was classified as bcl-2 positive if more than 10% of the tumor cells were stained.

Immunohistochemistry for p53 Protein. For p53 immunohistochemistry, polyclonal antibody CM-1 (diluted 1:1000; Novocastra Laboratories, Newcastle upon Tyne, United Kingdom) and biotinylated antirabbit antibody (1:400; Dakopatts) were used. The reaction was visualized by an avidin-biotin complex (Dakopatts) and diaminobenzidine-hydrogen peroxide. As a positive control, we used a lung tumor demonstrated previously to show a strong nuclear positivity for p53 protein (19). p53-positive cases were divided into five groups: 0, no nuclei positive; 1, <1% of the nuclei positive; 2, 1—5%; 3, 6—10%; 4, 11—40%; and 5, >40%.

Statistical Analysis. The statistical analyses were performed with the SPSS for Windows program package (SPSS, Chicago, IL). Data are presented as mean ± SE. The significances of the associations were determined by using the χ2 test, Fisher’s exact probability test, or two-tailed t test. Univariate and multivariate analyses of survival data were undertaken by using survival curves and applying the Kaplan-Meier method with log rank analysis and the Cox regression model. The statistical analysis was performed from material of 71 squamous cell carcinomas and adenocarcinomas; the three small cell carcinomas and one large cell carcinoma were excluded. P < 0.05 was considered significant.

RESULTS

Morphological Features of Apoptosis in Lung Carcinoma and Nonneoplastic Tissues. Labeling of the 3' ends of the fragmented DNA made it easy to distinguish the apoptotic cells and bodies from the surrounding cells, although the cytoplasmic boundaries of the cells were often difficult to discern due to a light counterstaining. Some cells showed intensive labeling just underneath the nuclear envelope, indicating a condensation of chromatin at the early stages of apoptosis (Fig. 1A). In cells that had proceeded further on the pathway of apoptosis, the nuclear labeling was more uniform, with the fragmented chromatin being distributed throughout the nucleus (Fig. 1B). The apoptotic cells usually had small nuclei and condensed cytoplasm. Membrane-bound apoptotic bodies were detected frequently in the tissue sections. Recently emerged apoptotic bodies appeared in small groups with two or three particles in close apposition to each other (Fig. 1C). There were also more numerous and smaller membrane-bound fragments scattered throughout the tissue. They probably represent the remnants of the nuclei. By the in situ labeling, it was possible to detect apoptotic bodies as small as 0.5 µm in diameter. In general, due to faint counterstaining, it was impossible to define whether the bodies were intracytoplasmic, e.g., phagocytosed by neighboring cells, or extracellular. Generally, the immunofluorescence technique provided slightly better resolution for the detection of apoptotic bodies. The peroxidase method seemed, however, to provide a better assessment of the background histology, more convenient for determining the apoptotic indexes (Fig. 1, D—F).

Apoptotic Indexes in Lung Carcinoma and Nonneoplastic Cells. The extent of apoptosis in different histological types of lung carcinomas and nonneoplastic tissues is shown in Tables 1 and 2. The absolute and relative numbers of apoptotic bodies showed a significant correlation with the number of apoptotic cells in the tumors (r = 0.80; P < 0.0001; and r = 0.81; P < 0.0001, respectively). In each tumor, the number of apoptotic bodies was roughly 2-fold greater than the number of apoptotic cells, whereas in the nonneoplastic...
controls, the ratio between apoptotic cells and bodies was roughly 1:1. There were notable differences in the apoptotic indexes in different areas and cells examined, which are shown in Tables 1 and 2. Cultured, untreated peripheral blood lymphocytes showed a fairly high and variable extent of apoptosis (range, 3–15%), whereas treatment with dexamethazone increased the extent of apoptosis approximately 2-fold (range, 15–30%). By electron microscopy, lymphocytes going through apoptosis showed typical condensation of the nuclear chromatin (not shown).

**PCNA and Necrosis in Lung Carcinoma.** The PCNA positivity in the positive lymph node control and in lung tumors was strictly nuclear. All tumors showed a high proliferation, with squamous cell carcinomas showing significantly more often more than 50% of the nuclei positive for PCNA than adenocarcinomas ($P = 0.009$ by Fisher’s exact probability test; Table 3). The cells expressing PCNA were, in general, poorly differentiated and were seen mostly in the periphery of the tumor cell islands, especially in squamous cell carcinomas, in which the peripheral cells were intensively positive for PCNA, whereas the keratinized cells in the middle of the tumor remained negative. The only large cell carcinoma in this material and all the small cell carcinomas had more than 50% of the nuclei positive for PCNA. In squamous cell carcinoma, a high percentage (>50%) of the nuclei positive for PCNA was found significantly more often in high-grade (grade III) than in low-grade tumors (grades I-II;
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Table 1 Apoptosis in lung carcinoma

<table>
<thead>
<tr>
<th></th>
<th>Apoptotic cells (mean ± SE)</th>
<th>Apoptotic bodies (mean ± SE)</th>
<th>Apoptotic cells and bodies (mean ± SE)</th>
<th>No. of cases studied</th>
</tr>
</thead>
<tbody>
<tr>
<td>Squamous cell carcinoma</td>
<td>1.47 ± 0.20</td>
<td>3.15 ± 0.49</td>
<td>4.62 ± 0.67</td>
<td>47</td>
</tr>
<tr>
<td>Adenocarcinoma</td>
<td>1.36 ± 0.23</td>
<td>2.21 ± 0.51</td>
<td>3.58 ± 0.74</td>
<td>24</td>
</tr>
<tr>
<td>Small cell carcinoma</td>
<td>6.83 ± 1.40</td>
<td>12.33 ± 4.43</td>
<td>19.17 ± 4.35</td>
<td>3</td>
</tr>
<tr>
<td>Large cell carcinoma</td>
<td>0.20</td>
<td>0.70</td>
<td>0.90</td>
<td>1</td>
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Table 2 Apoptotic indexes (%) in lung carcinoma and nonneoplastic control tissues

<table>
<thead>
<tr>
<th></th>
<th>Apoptotic index (%)</th>
<th>Apoptotic bodies (%)</th>
<th>Apoptotic cells and bodies (%)</th>
<th>No. of cases studied</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lung carcinomas</td>
<td></td>
<td></td>
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</tr>
<tr>
<td>Squamous cell carcinoma</td>
<td>0.38 ± 0.05</td>
<td>0.81 ± 0.14</td>
<td>1.20 ± 0.19</td>
<td>47</td>
</tr>
<tr>
<td>Adenocarcinoma</td>
<td>0.48 ± 0.08</td>
<td>0.75 ± 0.17</td>
<td>1.24 ± 0.24</td>
<td>24</td>
</tr>
<tr>
<td>Small cell carcinoma</td>
<td>0.92 ± 0.30</td>
<td>1.73 ± 0.71</td>
<td>2.65 ± 0.90</td>
<td>3</td>
</tr>
<tr>
<td>Large cell carcinoma</td>
<td>0.07</td>
<td>0.17</td>
<td>0.24</td>
<td>1</td>
</tr>
<tr>
<td>Nonneoplastic controls</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bronchial epithelium</td>
<td>0.02 ± 0.02</td>
<td>0.04 ± 0.01</td>
<td>0.06 ± 0.02</td>
<td>11</td>
</tr>
<tr>
<td>Serous and mucous glands</td>
<td>0.01 ± 0.01</td>
<td>0.00 ± 0.00</td>
<td>0.01 ± 0.01</td>
<td>7</td>
</tr>
<tr>
<td>Alveolar macrophages</td>
<td>1.35 ± 0.14</td>
<td>0.43 ± 0.12</td>
<td>1.78 ± 0.23</td>
<td>6</td>
</tr>
<tr>
<td>Lung interstitial cells</td>
<td>0.13 ± 0.02</td>
<td>0.11 ± 0.03</td>
<td>0.24 ± 0.04</td>
<td>6</td>
</tr>
<tr>
<td>Hyperplastic lymph node</td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Germinal center</td>
<td>1.50 ± 0.26</td>
<td>2.34 ± 0.11</td>
<td>3.84 ± 0.23</td>
<td>4</td>
</tr>
<tr>
<td>Extral follicular area</td>
<td>0.08 ± 0.03</td>
<td>0.06 ± 0.01</td>
<td>0.14 ± 0.02</td>
<td>6</td>
</tr>
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</table>

P = 0.04). Table 4 shows the extent of necrosis in lung carcinoma. Necrosis was seen more frequently in squamous cell carcinomas than in adenocarcinomas (P < 0.00001 by Fisher’s exact test; Table 4).

Immunoreactivity for p53 and bcl-2 in Lung Carcinoma. Fifty-three % of the squamous cell carcinomas and 50% of the adenocarcinomas showed p53-positive nuclei. The large cell carcinoma and one small cell carcinoma were p53 positive (Table 5). In squamous cell carcinomas, most of the p53-positive nuclei were seen in the peripheral cells, whereas the keratinized areas of the tumor remained negative. p53-stained nuclei were never seen in the adjacent normal structures.

bcl-2 positivity was seen only in the cytoplasm of the cells. In hyperplastic lymph nodes used as controls, the staining for bcl-2 was restricted to the lymphocytes outside of the germinal centers. Seventeen of 75 tumors (22.7%) were bcl-2 positive. The bcl-2 positivity was more frequent in squamous cell carcinomas [13 (27.7%) of 47 tumors] than in adenocarcinomas [2 (8.3%) of 24 tumors]. Two of three small cell carcinomas were bcl-2 positive. The large cell carcinoma was bcl-2-negative. No association was found between the expression of p53 and bcl-2 protein in lung tumors.

Apoptosis in Relation to Cell Proliferation and Necrosis. In each tumor, apoptotic cells were found scattered evenly throughout the tumor with no preferential accumulation, e.g., at the areas of necrosis. Apoptotic indexes were higher in general in tumors with more than 50% of the nuclei positive for PCNA (mean, 1.27 ± 0.20%) than in tumors with less than 50% of the nuclei positive for PCNA (mean, 1.13 ± 0.22%). Tumors with more than 20% necrosis showed slightly higher apoptotic indexes (mean, 1.25 ± 0.22%) than less necrotic or nonnecrotic tumors (mean, 1.16 ± 0.20%), but neither of these achieved statistical significance.

Apoptosis in Relation to p53 and bcl-2 and to Other Investigated Parameters. p53-positive tumors (more than 5% of the nuclei positive for p53) showed slightly higher apoptotic indexes (mean, 1.28 ± 0.21%) than p53-negative tumors (mean, 1.16 ± 0.20%). This difference was not statistically significant.

bcl-2-positive tumors had lower apoptotic indexes (mean, 0.83 ± 0.16%) than bcl-2-negative tumors (mean, 1.31 ± 0.18%; P = 0.13). In the extral follicular area of the lymph nodes, used as a positive control for bcl-2, the number of apoptotic cells or bodies was extremely low. On the other hand, in the bcl-2-negative germinal centers, the percentage of apoptotic cells and bodies was very high (data not shown).

In general, the patients younger than 60 years showed slightly higher apoptotic indexes in their tumors (mean, 1.16 ± 0.23%) than patients older than 60 years (mean, 1.00 ± 0.14%). The percentage of apoptotic cells in lung carcinoma was significantly higher in high-grade (grade III) carcinomas than in low-grade carcinomas (grades I-II, P = 0.019). There was no association between the TNM status of the tumors and their apoptotic index.

PCNA, p53, and bcl-2 in Association with Tumor Necrosis and Other Investigated Parameters. Interestingly, more than 50% of the nuclei positive for PCNA were found significantly more often in
occurrence of tumor necrosis increased the risk 2.3-fold (P = 0.02; patients with less necrotic or nonnecrotic squamous cell carcinoma 95% CI, 1.80—8.40 and 1.10—4.90, respectively). Thus, both factors a 3.9-fold risk for a shortened survival (P = 0.0005), whereas the (P < 0.04; Fig. 3B). By multivariate analysis, p53-positivity showed more than 20% necrosis had significantly shorter survival than pa

The expression of PCNA or bcl-2, relative amount of tumor necrosis, no. of 20 29 16 8 275 carcinomaI1Total

The main purpose of this study was to determine the extent of apoptosis in lung carcinoma and to evaluate it as a prognostic marker. Our working hypothesis was that a high level of apoptotic activity, suggesting slower tumor growth, would be linked to better survival in lung carcinoma. However, the opposite turned out to be true; enhanced apoptosis was associated with shortened survival. Also, Lipponen and Aaltomaa (20) have provided data showing a high apoptotic index to be associated with a poor prognosis in transitional cell carcinoma of the urinary bladder. These results suggest, that at least in some tumors, high apoptotic activity is an ominous prognostic sign. As reflected against the backdrop of proliferation and apoptotic rates dictating the net growth rate of the tumor, the results emphasize strongly the need to develop methods for determining measures to assess the duration of apoptosis, a critical determinant of apoptosis.

Also, p53 positivity showed a clear association with survival; patients with p53-positive tumors had significantly poorer prognoses. This is in line with some previous results on lung tumors (12—15). On the other hand, there are also studies that have not found any differences in survival between p53-positive and p53-negative lung tumors (21).

The detection of apoptotic cells and bodies by using normal light microscopy is based on several morphological features previously presented in the literature (22, 23). These include condensation of chromatin and cytoplasm and fragmentation of the cells, which leads to the appearance of membrane-bound apoptotic bodies, which contain, e.g., remnants of nuclei, cytoplasm, and cell organelles. Due to their small size, apoptotic bodies can be difficult to detect in tissue sections by using conventional light microscopy. By using the 3' end labeling of the fragmented DNA, apoptotic bodies as small as 0.5 μm in diameter could be counted accurately, allowing a more precise evaluation of the extent of apoptosis.

The time needed from an onset to a completion of apoptosis varies from one cell type to another. According to Kerr et al. (22), apoptotic bodies may form and disappear in 24 h. In normal liver, the duration of the visible stages of apoptosis is less than 3 h (24). Currently, there are no established techniques to determine the duration of apoptosis in tumor tissues. Some inferences can be made, however, on the basis of the relative occurrence of various types of apoptotic bodies, thought to represent various stages of apoptosis (24). In the present study, the ratio between apoptotic cells and bodies in lung carcinoma was roughly 1:2, whereas in control tissue it was 1:1. This can be taken to indicate a higher turnover rate of apoptosis in tumors when compared with nonneoplastic tissue.

In view of the notion that the growth rate of the tumor reflects the net gain of cells, it is possible, in theory, to make predictions about the growth rate on the basis of the observed rates of proliferation and apoptosis. However, even small differences, e.g., in the duration of

<table>
<thead>
<tr>
<th>Table 4 Necrosis in lung carcinoma</th>
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<tbody>
<tr>
<td>% Necrosis, no. of cases</td>
</tr>
<tr>
<td>0%</td>
</tr>
<tr>
<td>Squamous cell carcinoma</td>
</tr>
<tr>
<td>Adenocarcinoma</td>
</tr>
<tr>
<td>Small cell carcinoma</td>
</tr>
<tr>
<td>Large cell carcinoma</td>
</tr>
<tr>
<td>Total no. of carcinomas</td>
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</table>

a Squamous cell carcinomas contain significantly more often necroses of any percentage than adenocarcinomas (P < 0.00001).

<table>
<thead>
<tr>
<th>Table 5 p53 positivity in lung carcinoma</th>
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<tbody>
<tr>
<td>No. of p53-positive cases</td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td>Squamous cell carcinoma</td>
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<tr>
<td>Adenocarcinoma</td>
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<td>Small cell carcinoma</td>
</tr>
<tr>
<td>Large cell carcinoma</td>
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<td>Total no. of carcinomas</td>
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a No positive nuclei; (+), <1% of the nuclei positive; ++, 1–5% of the nuclei positive; ++++, 11–40% of the nuclei positive; ++++, >41% of the nuclei positive.

were independent prognostic markers. In adenocarcinoma, none of the parameters investigated was associated with the survival rate. The TNM status did not have any correlations with patient survival.
apoptosis, may have dramatic effects on the tumor growth. Thus, even within fairly uniformly fatal cancers, such as lung cancers, no straightforward relationships between the proliferation rate and the numbers of apoptotic cells and bodies can be expected. Indeed, in the present study, we did not find any association between the expression of PCNA and the extent of apoptosis. There was, however, an association between PCNA positivity and the occurrence of tumor necrosis. The same association has been shown to exist in stromal tumors of the stomach (25) and in synovial sarcomas (26).

*p53* is an important regulator of apoptosis, convincingly shown, e.g., in experiments in which extensive apoptosis was induced on transfection of *p53* into *p53*-deficient myeloid leukemic cells (6). Mutated *p53*, on the other hand, has lost its capacity to induce apoptosis. Because immunohistochemical detectability is commonly held to represent accumulation of mutated *p53* due to its extended half-life, one might expect to see an inverse relation between the extent of apoptosis and the occurrence of *p53*-positive cells, as seen, for example, in Wilms' tumors (27). In bladder cancer, the relationship has been shown to be the opposite; *p53* positivity was associated with a high extent of apoptosis (20). In this study, 53.2% of the squamous cell carcinomas and 50% of the adenocarcinomas showed positive immunohistochemistry for *p53*, indicative of abnormal accumulation of *p53* protein. This is well in line with some previous studies, in which 59% of the squamous cell carcinomas and 52% of the adenocarcinomas were found to be *p53* positive (21). There was not, however, any association between the extent of apoptosis and the occurrence of *p53*-positive cells.

There was much more necrosis in lung tumors classified as *p53* positive than in *p53*-negative lung tumors. A similar association has been shown previously in mammary ductal carcinoma *in situ* (28). At...
least in the brain, in cases of ischemia, the accumulation of p53 protein accompanies necrotic cell death (29). It is not immediately clear whether it is the necrosis that causes p53 protein to accumulate or vice versa.

bcl-2 is also an important regulator of apoptosis, known to inhibit apoptosis. We found 28% of the squamous cell carcinomas and 8% of the adenocarcinomas to be bcl-2 positive. Similar frequencies of bcl-2 positivity have been reported for lung carcinoma before (16). In breast cancer, bcl-2 positivity has been found to be associated with a higher grade of the tumor, p53 negativity, lack of tumor necrosis, and a favorable outcome (30). In our study, we found bcl-2 positivity to be associated with a greater extent of tumor necrosis but not with p53 negativity or longer survival of the patients.

Our data show that the extent of apoptosis seems to be independent of the expression of p53, PCNA, and bcl-2 in non-small cell lung carcinoma. These data also demonstrate clearly that both enhanced apoptosis and p53 positivity, predicting shortened survival, are independent prognostic markers in non-small cell lung carcinoma.

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