A High Number of Tumor-infiltrating Lymphocytes Are Associated with a Small Tumor Size, Low Tumor Stage, and a Favorable Prognosis in Operated Small Cell Lung Carcinoma

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ABSTRACT

Tumor-infiltrating lymphocytes, apoptosis, and angiogenesis have a pivotal role in tumor growth control. This study was undertaken to analyze the associations of these factors and their role in the prognosis, defined as survival time, of 56 patients operated on for small cell lung carcinoma (SCLC). Immunohistochemically detected T cells and macrophages were the most abundant tumor-infiltrating lymphocytes in SCLC, whereas the number of B cells was small. There was a trend in the number of intratumoral cytotoxic/suppressor CD8 cells that were associated with the extent of apoptotic bodies in SCLC, as measured by in situ 3'-end labeling of apoptotic DNA. A high number of intratumoral T cells and CD8 cells were associated significantly with a low tumor size (<3 cm) and low tumor stage (stages I–II). A high number of intratumoral macrophages were associated with a low tumor stage and angiogenesis, as measured by microvessel density. A high number of T cells, CD8 cells, and macrophages and a low tumor size (<3 cm) were prognostic markers predicting favorable survival time of the patients with SCLC.

INTRODUCTION

SCLC is the most rapidly progressing form of lung cancer, having the highest growth fraction and shortest doubling time (1). In addition, SCLC has proven to be distinctive from all other types of lung carcinoma in its initial sensitivity to chemotherapy. However, even with aggressive treatment modalities, most patients with SCLC will die of the metastasized disease in less than 2 years after the initial diagnosis.

Apoptosis is a genetically regulated active process whose purpose is to abolish cell populations in both physiological and pathological processes (2). Impaired apoptosis, whether attributable to expression of oncogenes or mutations of tumor suppressor genes, leads to an uncontrolled accumulation of malignant cells and the eventual formation of cancer (3, 4). Apoptosis also has a fundamental role in immune response such as deletion of immune cells recognizing self-antigens and in cytotoxic killing (5). Recent evidence suggests that T-cell function in a tumor microenvironment can be modulated through a FasR/FasL apoptotic pathway (6). FasL-bearing cytotoxic T cells are able to promote apoptosis in FasR-bearing target cells. Interestingly, functional FasL is expressed in several tumor types including lung carcinoma (7–9). This suggests that FasL-expressing tumor cells actively destroy FasR-bearing TILs and they, thereby, may contribute to tumor progression.

TILs participate in tumor growth control not only through T-cell cytotoxicity but also by producing soluble mediators, i.e., growth-stimulating and -inhibiting cytokines (10). Moreover, tumor cells themselves can also produce these cytokines (11). The majority of TILs in human tumors consist of T cells, suggesting an activation of host defense mechanism (12). Tumor-host interactions have been shown to play a significant role in predicting the disease outcome in several human tumors (12–15). However, in lung carcinoma, the role of TILs is indistinct, and prognostic results are controversial (16, 17).

There is substantial proof that angiogenesis is vital for tumor growth and metastasis (18). Several factors are known to stimulate new vessel formation, of which basic fibroblast growth factor and vascular endothelial growth factor are the most commonly expressed in human tumors (19). It has been suggested that some of these growth factors are under the control of the p53 tumor-suppressor gene (20, 21). Moreover, there is evidence that inhibition of angiogenesis limits tumor growth by promoting apoptosis (22). In non-SCLC, tumor vascularity has been found to be an independent prognostic factor and important for the formation of hematogenous metastases (23–25).

Defective apoptosis, changes in cell-mediated immunity, and the formation of new microvessels have been proposed as significant factors in the development and growth of lung carcinoma. Previously, a low number of some TILs (16, 17), accelerated apoptosis (26), and a high number of microvessels (23–25) have been associated with a shortened survival time of the patients with non-SCLC. This study was undertaken to analyze the relationships of TILs, apoptosis, and angiogenesis in vivo and their significance on the prognosis, defined as survival time, of 56 patients operated on for SCLC. To our knowledge,
MATERIALS AND METHODS

Tumor Material. A total of 56 patients with SCLC (5 women and 51 men, ages 44–77 years) were included in the series. The patients had been surgically treated at Oulu University Hospital during the years 1975–1998. All surgical samples were obtained for therapeutic and diagnostic purposes and hence were not subjected to any kind of chemotherapy. The removed lungs or lobes containing the tumor were fixed intrabronchially with 10% buffered formalin overnight, and the tumor samples were embedded in paraffin. SCLC was diagnosed histologically according to the WHO International Histological Typing of Lung Tumors (27). A representative tissue block from each tumor was selected for the labeling of apoptotic cells and for the immunohistochemical stainings. Clinical follow-up and other data of the patients were collected from the hospital records. Of the 56 SCLC tumors, 37 (66%) were stage I–II tumors and 19 (34%) were stage III–IV tumors. There were 5-year follow-up data available from 42 patients, 35 (64%) of whom died of the disease.

Immunohistochemical Staining of TILs. For immunophenotypic analysis of inflammatory cells in lung carcinoma samples, monoclonal mouse antibodies UCHL-1, L26, PGM-1 (DAKO, Glostrup, Denmark), and CD8 (Novocastra Laboratories, Newcastle upon Tyne, United Kingdom) were used. UCHL-1 (anti-CD45) labeled resting T cells within both the CD4 and CD8 subsets and mature activated T cells. CD8 labeled cytotoxic suppressor T cells. L26 (anti-CD20) was directed against an antigen present on the majority of B cells. CD4 and CD8 subsets and mature activated T cells. CD8 labeled cytotoxic suppressor T cells. L26 (anti-CD20) was directed against an antigen present on the majority of B cells. PGM-1 stained macrophages. All of these antibodies were used at a dilution of 1:50.

Immunophenotypic Analysis of TILs. An area containing an average of 700 tumor cells (range, 100-1200) in a minimum of five HPFs was evaluated in each case, and the absolute numbers of intratumoral T, CD8, and B lymphocytes, and of macrophages were calculated per HPF, avoiding necrotic areas (×40 objective; diameter of the field, 400 μm). The average number of each inflammatory cell type per HPF is given.

Microvessel Staining and Evaluation. Microvessels in the tumor tissue were detected by immunohistochemical staining, with antifactor-VIII-related antigen polyclonal antibody A0082 (dilution of 1:200), which labels the vascular endothelium (DAKO).

The avidin-biotin complex staining method was used for all of the immunohistochemical stainings, with diaminobenzidine as a chromogen. Finally, all of the sections were lightly counterstained with hematoxylin.

The absolute number of microvessels in the tumors was evaluated from five consecutive HPFs, avoiding necrotic areas. Finally, the average number of microvessels per HPF is given. MD was considered a continuous variable, and a median value of nine microvessels per HPF was used as a limit between a low and high MD.

Detection of Apoptotic Cells and Bodies. The 3’-end labeling of apoptotic DNA was performed by using an ApopTag in situ apoptosis detection kit (Oncor, Gaithersburg, MD) and by following the manufacturer’s instructions, with a few modifications as described previously (26).

The extent of apoptosis was detected using two indices. The first index involved the number of apoptotic cells and bodies, counted separately, per HPF, with a mean of 10 HPFs, and the second index expressed apoptotic cells and bodies given as a percentage of the total number of tumor cells within a HPF (percent index). A hyperplastic lymph node showing an increased number of apoptotic cells within germinal centers served as a positive control, and a tumor sample shown previously to exhibit a high apoptotic index (28) when the TdT-enzyme was omitted served as a negative control.

Statistical Analysis. The statistical analyses were performed with the SPSS (Chicago, IL) for Windows program package. The significance of the associations was determined using Fisher’s exact test, the two-tailed t test, and linear regression. Univariate analysis of the survival data was performed using survival curves that applied the Kaplan-Meier method with log rank analysis. A probability of P < 0.05 was considered statistically significant.

RESULTS

Quantity of TILs and Their Associations. The mean numbers of TILs/HPFs were as follows: T cells, 14.7 (range, 0.20–62.4); CD8 cells, 10 (range, 0.15–45.0); B cells, 1.30 (range, 0–14.4); and macrophages, 23.1 (range, 1.20–66.2; Table 1). Sixty-eight percent of all T cells were CD8 cells. There were 24 (43%) of 54 cases without any intratumoral B cells in SCLC.

There was a statistically significant positive association between T cells and B cells in SCLC (P < 0.0005 and P = 0.02 by Pearson’s test). Both T cells and CD8 cells were also associated with macrophages in SCLC (P = 0.003 and P = 0.005 by Pearson’s test).

TILs in Relation to Apoptosis. In SCLC, a high number of intratumoral CD8 cells was detected more often in tumors with a high percentage of apoptotic bodies, although this association did not reach a statistical significance (P = 0.095 by Fisher’s exact test). No association was found between CD8 cells and apoptotic index or apoptotic cells alone in SCLC.

TILs in Relation to Tumor Diameter and Stage. An association was found between the number of T cells and CD8 cells and the diameter of the tumors. Namely, smaller tumors (diameter < 3 cm) displayed high numbers of intratumoral T
cells and CD8 cells significantly more often than large tumors did \((P = 0.05\) for both by Fisher’s exact test). Also the numbers of intratumoral T cells and CD8 cells were significantly higher in stage I–II tumors than in stage III–IV tumors \((P < 0.002\) and \(P < 0.05\), respectively). Interestingly, the number of intratumoral macrophages was also significantly higher in stage I–II tumors than in stage III–IV tumors \((P < 0.02)\).

**TILs, Tumor Diameter, and Tumor Stage as a Prognostic Marker in SCLC.** The patients with SCLC showing a high number of intratumoral T cells \((\geq 7.8/\text{HPF})\) and CD8 cells \((\geq 5.8/\text{HPF})\) had a significantly better survival time than those with a lower number of T cells \((<7.8/\text{HPF})\) or CD8 cells \((<5.8/\text{HPF}; P = 0.007\) and \(P = 0.02\) by log rank; Figs. 1 and 2). A high number of intratumoral macrophages \((\geq 19.0/\text{HPF})\) was also associated with a favorable survival \((P = 0.05\) by log rank; Fig. 3). The number of intratumoral B cells was not significantly associated with survival \((P = 0.634\) by log rank).

The patients with SCLC in whom the tumor diameter was smaller than 3 cm had a significantly better survival time than those in whom the tumor diameter was 3 cm or larger \((P = 0.036)\). However, the patients with SCLC in whom the tumor stage was I–II had an only slightly, but not statistically significant, better survival time than those in whom the tumor stage was III–IV \((P = 0.061)\).

**MD in Relation to Other Parameters.** There was a positive, although not statistically significant, association between intratumoral macrophages and MD in SCLC (Fig. 4). For example, a higher MD was detected more often in tumors with a high number of intratumoral macrophages \((P = 0.06\) by Fisher’s exact test). No associations could be found between MD and apoptosis in SCLC. Moreover, MD did not associate with tumor stage, tumor diameter, or the survival of the patients with SCLC.

**DISCUSSION**

TILs are known to have diverse functions in the tumor microenvironment. They produce soluble cytokines that regulate the proliferation and metastatic activity of tumor cells, promote angiogenesis, and participate in host defense mechanisms against tumor cells (5, 24, 29). It has become increasingly clear that cellular functions of TILs, i.e., cytotoxic T-cell killing and apoptosis, have a pivotal role in tumor growth control (30–32).

T cells and macrophages were the most commonly detected TILs in SCLC with a minority of B cells, which is in line with previous studies presented by Kerr et al. (17) and Watanabe et al. (16). The number of TILs has been associated with tumor type and degree of differentiation in lung carcinoma. It has been highest in well-differentiated squamous cell lung carcinoma and lowest in poorly differentiated small cell carcinoma (16, 17). Compared with our previous results (33), the overall number of TILs in SCLC is less than half of that detected in LCLC. Moreover, CD8 cells represent a much larger proportion of T cells in SCLC (68%) than in LCLC (43%; Ref. 33), suggesting a more important role of CTLs in host-tumor interaction in SCLC. However, the absolute numbers of T cells and CD8 cells in LCLC (33) were significantly higher than those in SCLC. This is confounding because in our previous study of LCLC, a shortened survival was associated with a relatively small number of B cells rather than with the larger number of T cells and CD8 cells that were found (33). This raises a question about the functional significance of the smaller number of T cells and CD8 cells found in the present study of SCLC. This apparent paradox warrants additional studies.

A high number of TILs has been associated with a favor-
able prognosis, particularly in fast-growing tumors, such as in nodular-type bladder cancer (13), fast-growing breast tumors (34), and melanoma (35). In lung carcinoma, the occurrence of TILs seems to point to a better chance of patient survival. According to Kerr et al. (17), T cells are associated with a better survival rate for the patient with non-SCLC. Riemann et al. (36) and our previous results (33) show that B cells are a more significant prognostic marker in non-SCLC than are T cells. In line with Kerr et al. (17), we demonstrate that T cells and cytotoxic CD8 T cells predict better survival, suggesting an active host defense mechanism in SCLC.

CTLs can specifically induce death in the target cell by at least two mechanisms: (a) mediated by direct exocytosis of granules that contain perforin and granzymes; and (b) mediated by signaling by FasL (5). Cooperation between secreted granzymes and perforin has been shown to cause typical cytotoxic lymphocyte-induced lysis and DNA fragmentation (37). In the Fas-mediated pathway, engagement of cytotoxic lymphocyte
membrane ligand (FasL) with an apoptosis-inducing target cell surface receptor (FasR) triggers apoptosis of the target cell (38). The functional expression of FasL on tumor cells is a mechanism of tumor escape from immunological detection of TILs that has been proposed by several groups (6, 8, 32). In addition, lung carcinoma has been shown to express FasL both in vitro and in vivo (7). This enables lung carcinoma cells to counterattack against Fas-sensitive TILs and thus contribute to tumor growth. On the other hand, an impaired FasR/FasL apoptotic pathway may contribute to immunologically nonreactive TILs being detected in lung carcinoma (39). We found an association between cytotoxic CD8 T cells and the occurrence of apoptotic bodies in SCLC, suggesting at least some involvement of an active FasR/FasL- or a perforin-mediated apoptotic pathway. Although our 3' end-labeling technique does not allow for verification of the origin of either the apoptotic cells or especially the apoptotic bodies, because of the positive association of apoptotic bodies and CTLs, it is likely that apoptotic bodies that represent dying tumor cells were attacked by cytotoxic T cells. Another possibility would be that apoptotic bodies are remains of activated T cells resulting from the down-regulation of the immune response. To verify this, it would be essential to evaluate the expression of FasL and FasR and their association to apoptosis and TILs in these tumors.

Macrophages have been shown to be cytotoxic toward tumor cells (29) and to produce growth-inhibitory factors (11). In accordance with our previous results (33) and results presented by Kerr et al. (17), a high number of macrophages was associated with a better survival rate for the patients with SCLC. Another function of macrophages is to promote angiogenesis by secreting proangiogenic cytokines and/or enzymes that degrade the extracellular matrix (40). In SCLC, a high MD was associated with a high number of intratumoral macrophages, which is in accordance with our previous results in LCLC (33).

Despite the undoubtedly important role of angiogenesis in tumor growth and in the formation of metastases (11, 18), MD was not associated with metastatic status, tumor size, or prognosis in SCLC. Previously in non-SCLC, microvessel count has been associated with metastasis (11, 23). It is suggested, however, that lung carcinomas in general use preserved alveolar basement membranes for their local

Fig. 4 A set of examples of TILs and microvessels in SCLC. A, intratumoral T cells in SCLC detected by monoclonal UCHL-1 antibody. B, intratumoral cytotoxic T cells in SCLC detected by monoclonal CD8 antibody. C, intratumoral macrophages in SCLC detected by monoclonal PGM-1 antibody. D, intratumoral microvessels in SCLC detected by polyclonal antifactor-VIII-related antigen (A–D, ×280).
spread and are thus not dependent on the new microvessel formation (41). Fontanini et al. (21) reported that angiogenesis in non-SCLC is under p53 tumor-suppressor gene control. Their results suggest that mutated p53 protein promotes angiogenesis through VEGF expression. This would lead to abrogation of p53-mediated apoptosis. However, we found no association with apoptosis and MD in SCLC.

We conclude that the high occurrence of intratumoral T cells, cytotoxic CD8 cells, and macrophages, and a low tumor size predict a favorable survival time for the patients operated for SCLC. Furthermore, regulation of the FasR/FasL apoptotic pathway may be a key mechanism in tumor growth control in SCLC and, thus, it needs to be studied further.

REFERENCES


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