Local Concentrations of CC-Chemokine-Ligand 18 Correlate with Tumor Size in Non-small Cell Lung Cancer and Are Elevated in Lymph Node-positive Disease

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Abstract. *Background: The tumor microenvironment plays a critical role in tumor growth and spreading. Tumor-associated macrophages (TAM) make up a large proportion of the tumor mass and are one of the main producers of CC-chemokine ligand 18 (CCL18), which is believed to carry out important functions in the immunological interactions that promote tumor progression. Materials and Methods: Cytokines/chemokines were measured in bronchoalveolar lavage (BAL) from the tumor site and serum before and after resection in patients with proven non-small cell lung cancer (NSCLC). Results: CCL18 concentrations in BAL positively correlated with the radiologically determined tumor volume ($r=0.72$, $p=0.0003$) in NSCLC. In addition, tumors with lymph-node metastasis exhibited significantly higher CCL18 concentrations in BAL ($p=0.049$) than those without. Serum CCL18 concentrations did not differ significantly before and after tumor resection. Conclusion: The increased release of CCL18 with greater tumor size is most likely due to the accompanied growth of leukocyte infiltrate. With previous findings taken into account, this could be one factor contributing to tumor invasiveness and particularly lymphatic spread in patients with larger tumors.*

Lung cancer is the leading cause of cancer-related death and is usually diagnosed at an advanced stage, where lymph node/distant metastases are present. Only in about one-third of patients with non-small cell lung cancer (NSCLC) the disease

is diagnosed as localized, for which a complete resection is the treatment of choice and a cure is possible. Hence, in most cases systemic therapy is required, either as part of a multimodal treatment or as sole therapeutic option. Despite advances in individualized approaches and modern chemotherapeutic regimens, the course of the disease remains unfavorable (1-3).

It is widely acknowledged that the tumor microenvironment plays a critical role in tumor growth and spread, and in recent years, multiple therapeutic approaches have been developed based on this knowledge (4). In contrast to agents focusing on molecular targets such as the epidermal growth factor receptor (EGFR), and anaplastic lymphoma kinase (ALK), whose activity is confined to tumors with specific genetic alterations, immunotherapy is effective in various histological subtypes (5-8).

Tumor microenvironment consists of tumor cells, the leukocyte infiltrate and the tumor associated immunologic microenvironment. One of the key players, the tumor associated macrophages (TAMs) can make up a large fraction of the tumor mass (9). Depending on the tumor microenvironment, macrophages can be polarized into different functional states, of which the two extremes are known as the M1 and M2 phenotypes. M1 macrophages have been shown to carry out pro-inflammatory, cytotoxic and thus mostly anti-neoplastic effects, whilst the M2 phenotype is responsible for tissue remodeling and repair and is believed to be involved in the subversion of adaptive immunity and thus the immunological interactions that promote tumor growth and progression. TAMs in the tumor microenvironment are mostly found to have the M2 phenotype (10). TAMs secrete a specific pattern of cytokines and chemokines and are one of the main producers of CC-chemokine ligand 18 (CCL18) in this context (11, 12). CCL18 is responsible for the recruitment of naive T-cells and dendritic cells, and is capable of inducing regulatory T-cells. In the tumor microenvironment, it has been demonstrated to promote tumor spread and is believed to be one of the factors involved in the immune escape of tumor cells (13-17).

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To enable further insight into the interactions of TAMs and the tumor microenvironment, we conducted a study concerning CCL18 and associated cytokines involved in these immunological interactions and their correlation with clinical tumor biology in NSCLC.

Materials and Methods

In this prospective observational study, we enrolled 37 patients with proven NSCLC. The study was approved by our local Ethics Committee and registered as a clinical trial in the German Registry for Clinical Trials (DRKS-ID: DRKS00005415). All patients signed an informed consent form before participating in the study.

Inclusion criteria were the presence of a pulmonary nodule suspicious of primary lung cancer with no previous treatment in patients from whom bronchoalveolar lavage (BAL) samples were taken. Exclusion criteria were clinical or radiological signs of pneumonia, centrally located tumors, presence of other malignancies, previous radiation or chemotherapy, treatment with systemic or inhalative steroids and poor quality of the BAL sample (presence of ciliated epithelium, bloody samples and low recovery). In patients from whom serum samples were acquired, inclusion criterion was histologically proven NSCLC eligible for resection. BAL was performed (n=25) using flexible bronchoscopes as described elsewhere (18).

The cytokines and chemokines CCL18, indoleamine 2,3-dioxygenase (IDO), tumor necrosis factor (TNF), transforming growth factor β (TGF β), and interleukin (IL)-1 β , IL4, IL6, IL8, IL10, and IL12 were analyzed *via* ELISA according to the manufacturer's recommendations (R&D Systems, Minneapolis, MN, USA).

Serum samples were acquired from another cohort of patients with proven NSCLC which were eligible for tumor resection (n=12) preoperatively, first day after operation and on day 10 after operation. CCL18 concentrations were also analyzed using ELISA.

Radiological tumor volume was determined by board-certified radiologists (ID, LJK) according to the recommendations by the Fleischner Society (19).

Data were recorded in a database designed in Microsoft Office Excel (Microsoft, Redmond, WA, USA) and GraphPad Prism 6.03 (GraphPad Software Inc., La Jolla, CA, USA) was used for statistical analysis. The D'Agostino-Pearson omnibus normality test was performed. Normally distributed datasets were compared using Student's *t*-test. For non-normally distributed data, Mann-Whitney test was employed. Correlation was calculated using Spearman's *r*. Results were considered significant if the *p*-value was less than 0.05.

Results

Patients' characteristics. In total, 37 patients were included in the study. In all patients, histologically proven NSCLC was present, 24 classified as adenocarcinomas, 11 as squamous cell carcinomas and two as large-cell carcinomas.

CCL18 concentrations in BAL, but not in serum, correlate with radiologically determined tumor size. TAMs make up a large fraction of the leukocyte infiltrate and thus also of the tumor mass in solid tumors. To analyze possible increased immunological effects of this infiltrate with increased tumor

size, we radiologically determined the mean tumor volume and correlated it with concentrations in BAL of CCL18 and associated cytokines that influence the immune reaction in patients with malignant tumors.

We showed that CCL18 concentrations in BAL positively correlate with the radiologically determined tumor volume ($r=0.72$, $p=0.0003$) in NSCLC (Figure 1a). Comparison of the radiologically determined tumor size with pathological tumor size in tumors that were resected confirmed correct radiological measurements ($r=0.84$, $p<0.0001$).

High CCL18 concentration in BAL is associated with lymph node metastasis. CCL18 concentrations are known to be elevated in patients with lung cancer and this is believed to influence immunological interactions in the tumor microenvironment involving tumor progression and metastasis. Detailed analysis of CCL18 expression and clinicopathological features was performed. We found significantly higher CCL18 concentrations in patients with lymph node-positive ($2,585\pm1,961$ pg/ml) than in those with nodal-negative disease (988 ± 860 pg/ml; $p=0.049$) (Figure 1b). No systematic associations concerning other clinicopathological features, such as distant metastasis or clinical tumor stage, were observed.

Interestingly, we found no link between tumor volume ($r=0.15$, $p=0.64$) and serum CCL18 concentrations (Figure 2a). In addition, there was no difference in CCL18 concentrations in serum immediately and medium-term after tumor resection (Figure 2b).

None of the other measured cytokines showed any association with clinical findings. TGF β , IL8, IL12 did not significantly differ in the different groups regarding clinical or pathological tumor stage. IL1 β , IL10 IFN γ and TNF were also determined but their concentrations in most samples were below the detection limit of the ELISA used.

Discussion

TAMs are the most abundant immunological cells in the tumor microenvironment and have been associated with tumor progression in various solid tumors (15, 20, 21). They are also the main producers of CCL18, which has been shown to have important functions in immunological interactions in the tumor microenvironment and thus influence tumor growth and spread (14, 15). Previous studies on lung cancer demonstrated that the accumulation of CCL18-positive TAMs in pathological sections was associated with a poor prognostic outcome (22). Correspondingly, high serum concentrations of CCL18 in lung adenocarcinoma were also associated with an unfavorable prognosis (23).

In this study, we showed for the first time that CCL18 concentrations in BAL from the lung portion affected by tumor increases with tumor size. Based on the knowledge we have from previous studies, this finding most likely

Figure 1. Correlation of tumor size and CC-chemokine ligand 18 (CCL18) concentration in bronchoalveolar lavage (BAL) (A) and comparison of tumors with (N+) and without (N0) presence of lymph-node metastasis (B). CCL18 concentrations in BAL from the tumor site positively correlated with the radiologically determined tumor volume ($r=0.72$, $p=0.0003$). In tumors with lymph-node metastasis, CCL18 concentrations in BAL were significantly higher than in tumors without lymphatic spread ($p=0.049$).

Figure 2. Correlation of tumor size and CC-chemokine ligand 18 (CCL18) concentrations in serum (A) and comparison before and after resection of the tumor (B). Serum CCL18 concentrations did not correlate with tumor volume ($r=0.15$, $p=0.64$) and there were no differences before, immediately after and 10 days after tumor resection.

represents the growth of the leukocyte infiltrate represented by TAMs, which often make up a large fraction of the tumor, and their excessive CCL18 secretion (9, 10). CCL18 is known to carry out different immunomodulatory functions, including chemotaxis, and to participate in the homing of naive T-helper 4 cells and dendritic cells (24, 25). It has also been shown that it differentiates dendritic cells able to prime regulatory T-cells and thus induce tolerance (26). This action was suggested to lead to a failure of the immune system to adequately react to malignancies and kill tumor cells.

In the tumor microenvironment, CCL18 promotes epithelial to mesenchymal transition in different types of solid tumors, including NSCLC (13-15). In a recently conducted study by Shi and colleagues, it was shown that CCL18 modulates NSCLC cell invasion and migration *via* the nitrite reductase 1-engulfment and cell motility 1/dedicator of cytokinesis 180 (NIR1-ELMO1/DOC180) pathway and thus promotes tumor metastasis (22).

In NSCLC, tumor size is a prognosticator for the presence of lymph-node metastasis even in those with early disease

stage and smaller tumors (27). Interestingly, we recorded significantly higher CCL18 concentrations in BAL from those with primary lung cancer with lymph node metastasis compared to those with tumors without affected locoregional lymph nodes at the time of sampling. These findings are in line with multiple previous studies, where high CCL18 expression in pathological sections was associated with lymph-node metastasis and peritumoral lymphangiogenesis in different tumor types, including NSCLC (14, 22, 28). Moreover, in patients suffering from breast cancer, higher CCL18 levels in blood as well as in cancer stroma was associated with an increased occurrence of metastasis and tumor invasion, and reduced patient survival (29).

In conclusion, our data demonstrate an increased release of CCL18 with greater tumor size, which, considering previous data, is most likely due to the accompanied growth of the leukocyte infiltrate of which a large fraction is made up of TAMs. This could be one mechanism contributing to the clinical observation of a higher likelihood of lymphatic spread in patients with larger tumors.

Conflicts of Interest

There is no conflict of interest regarding the material discussed in this article.

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