The investigation of the mechanisms of lymphangiogenesis and the role of the lymphatic vessels in lung cancer

Ph.D. Thesis

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I. Introduction

The most frequent malignant tumor in the world is lung cancer with 1.35 million new cases per year. There is an increasing number of patients: in 1980 600 thousand cases were registered, in 2000 1.2 million. In Hungary we have 8000 new patients per year. The importance of lymphatic metastases is well recognized in cancer staging and treatment in patients with solid tumors such as lung cancer. However, the process of lymphatic invasion and metastasis to regional lymph nodes, and whether tumors promote lymphangiogenesis (ie, new lymphatic vessel growth) in a manner similar to angiogenesis, remain poorly understood. Recent advances in the biology and pathology of lymphangiogenesis have provided new insights into the field of lymphatic vascular biology. A major impediment to gaining such knowledge has been the lack of markers with which to isolate and study lymphatic endothelium. This has now been overcome with the discovery of molecules such as the lymphatic endothelial hyaluronan (HA) receptor LYVE-1, and podoplanin D2-40, which represent a new and valuable tools for such research. The discovery of two lymphangiogenic molecules, vascular endothelial growth factor (VEGF)-C and VEGF-D, and their receptor VEGFR3 changed the landscape for lymphatic studies by providing the critical molecular players and tools. In addition to members of the VEGF family, other growth factors such as FGF-2, Ang-1, Ang-2, and PDGFs have also been shown to induce lymphangiogenesis in vivo.

Endothelial progenitor cells have been shown to contribute to angiogenesis in various tumor models. Here, we have studied the relative contributions of bone marrow (BM)-derived endothelial progenitors and pre-existing lymphatic vessels to tumor lymphangiogenesis

II. Purpose

Although research of tumor-induced lymphangiogenesis has been eclipsed by the greater emphasis laid on the mechanisms of tumor vascularization, recent experimental evidence suggests that tumors can provoke lymphatic capillary growth by production of lymphangiogenic factors such as members of the vascular endothelial growth factor family, and that the activity of tumor-induced lymphangiogenesis is directly correlated with the extent of tumor spread to regional lymph nodes. However, it is still uncertain whether lymphatic spread is achieved through the formation and invasion of new lymphatics (tumor-induced lymphangiogenesis via sprouting) or whether tumors acquire their lymphatic vessels by co-option, as it was described recently by our group and other observers during development of tumor blood vasculature. In the case of NSCLC (Non Small Cell Lung Cancer), a putatively non-angiogenic growth pattern was observed. In this “alveolar type” of growth, tumor cells fill the alveoli, entrapping, but not destroying the alveolar walls with the co-opted capillaries. Furthermore, in the tumor cell nests circumscribed by the alveolar walls no neoangiogenesis is present.

Our hypothesis was that similarities may exist between hem- and lymphangiogenesis in NSCLC. Therefore, the objective of the fist part of our study was to clarify the role of the lymphatics in the progression of lung tumors. We have analyzed how NSCLC acquires its lymphatic network and investigated whether the extent of lymphangiogenesis may be related to the angiogenic phenotype (angiogenic vs. non-angiogenic) and/or to the risk of lymph node metastasis and to patient survival, using tumor samples obtained from NSCLC patients.

We already mentioned some mechanism of the formation of new lymphatic vessels in malignant tumors, like lymphatic vessel sprouting, or cooption of pre-excisting lymphatic vessels, but we have not talken about the LVEPCs (Lymphatic Vascular Endothelial

Progenitor Cells), which also play an important roll in this system. In the second part of our study we will discuss about the role of VEGFR3+ LVEPCs in the lymphangiogenezis of the SCLC (Small Cell Lung Cancer).
As part of the lymphangiogenic machinery, the newly identified bone marrow derived cell population, called lymphatic/vascular endothelial progenitor cells, has been shown to contribute to de novo lymphangiogenesis in human renal transplants, and more importantly, in experimental tumor systems. It is still unclear, however, whether LVEPCs participate in SCLC -induced lymph vessel growth. Nevertheless, since an analogous cell population (VEGFR2+ haemangiogenic endothelial progenitor cells, EPCs) has been demonstrated recently to have clinical significance in the haemangiogenic process of a wide range of human malignancies, including non-small cell lung cancer, we hypothesized that LVEPCs could be involved in the progression of human SCLC. Hence, using peripheral blood (PB) samples obtained from SCLC patients, we assessed the numbers of circulating LVEPCs by flow cytometry and investigated whether these numbers might be related to the levels of the key lymphangiogenic molecule vascular endothelial growth factor-C (VEGF-C) and/or to the risk of lymph node metastasis and to patient survival.

III. Materials and Methods

III/1. Clinical Data

In the first part of our study we had 103 patients with histopathologically defined NSCLC treated at the Department of Surgery, National Institute of Pulmonology, Budapest during January 1994–December 1997.

In the second part of our studies, to measure the number of circulating LVEPCs, peripherial blood samples were collected from 88 patients with limited disease (LD) SCLC before therapy. The control group included 32 individuals matched for age, gender, smoking status and spirometry test result.

III/2. Characterization of the lymphatic system by immunohistochemistry

For immunofluorescent staining, slices were incubated simultaneously with the appropriate primary (LYVE-1, D2-40, CD31, Ki67, VEGF-C) than with secondary antibodies. Finally, counterstaining was performed using methylene green.

III/3. Computer-assisted morphometric analysis of the lymphatic network

Morphometric parameters were determined by labeling of lymphatic vessels with anti-human LYVE-1 and anti-human D2-40/podoplanin. Three different tumoral and peritumoral regions were assessed separately for each section. These were: (I) the tumor centre; (II) the tumor periphery; and (III) the peritumoral host.

III/4. Enumeration of LVEPCs by flow cytometry from the peripheral blood of SCLC patients

We quantified the content of circulating LVEPCs by flow cytometric analysis. After appropriate gating, the number of CD34+/VEGFR3+ double positive cells were quantified and expressed as the number of cells per milliliter of blood.

III/5. Measuring the levels of VEGF-C in the peripheral blood of controls and patients with SCLC

For VEGF-C measurements, serum samples from all patients and controls were analysed. Levels of VEGF-C were quantified with the use of a commercial ELISA kit.

IV. Results

IV/1. Identification of lymphatic vessels in normal human lung tissue: Lyve-1 and D2-40/podoplanin antibody specificity

In the normal lung parenchyma, LYVE-1 (a lymph-specific receptor for hyaluronan) and D2-40 (specific for the lymphatic marker podoplanin) revealed a similar pattern. Positive staining was seen in thin-walled lymphatic vessels devoid of red blood cells. The distinction between blood and lymphatic vessels was further corroborated by double immunostaining for LYVE-1 or D2-40 and the panendothelial marker CD31. LYVE-1- and D2-40-positive lymphatic vessels were weakly CD31-positive. On the contrary, erythrocyte-containing CD31-positive blood vessels were LYVE-1- and D2-40-negative.

IV/2. Characterization of lymph vessels in human non-small cell lung cancer tissue

To characterize lymphatic capillaries in NSCLC, we carried out immunostaining with antibodies to LYVE-1 and D2-40. With both antibodies positive lymph vessels were always seen in the peritumoral lung and at the tumor periphery. With respect to LYVE-1 immunoreactivity, central tumor areas appeared devoid of immunoreactive vessels, except for non-angiogenic tumors growing

without host tissue destruction, where LYVE-1 expressing vessels were preferentially associated to co-opted intratumoral blood vessels. With respect to D2-40/podoplanin immunoreactivity, examination of the central tumor areas in destructively growing angiogenic tumors revealed that a minority of lymphatic vessels were in fact dispersed between the tumor cells in close contact with them. In these angiogenic tumors D2-40+ lymphatics were randomly distributed within the tumor mass.

IV/3. Lymphatic vessel density (LVD) and perimeter in human NSCLC

Because LVDs and lymph vessel perimeters of N1 and N2 tumor groups did not differ from each other significantly, node-positive and node-negative groups were established. Based on both LYVE-1 and D2-40 staining, in all the applied categories, LVD was highest in the peritumoral host tissue and the tumor periphery was more vascular than the tumor centre. Although evaluation of the LVDs by LYVE-1 or D2-40 reactivity in the peritumoral host tissue and in the tumor centre of NSCLCs revealed only a slight association with the presence of lymph node metastases, assessment of tumor periphery LVDs indicated a significant increase in the cases of both LYVE-1 and D2-40 in the lymph node-metastatic compared to the non-metastatic tumors. According to previous observers, based on CD31 immunoreactivity, NSCLCs were separated into angiogenic and non-angiogenic tumors. The patients with node-positive non-angiogenic cancer tended to have high tumor periphery LVD more often than those with node-negative disease, in the group of non-angiogenic tumors there was no statistically significant information from the LVD scores in any investigated part of the tumors. In the group of angiogenic tumors, tumor periphery LVD evaluated by both LYVE-1 and D2-40 immunostaining was significantly increased in lymph

node metastatic NSCLCs as compared with non-metastatic NSCLCs. Although quantification of LVDs by both the LYVE-1 and the D2-40 antibodies revealed a correlation between peritumoral LVDs and lymph node metastases in angiogenic tumors, this tendency proved to be statistically non-significant. No correlation was present between tumor centre LVD and N status in the angiogenic tumor group in the case of either LYVE-1 or D2-40.

IV/4. Patient survival

Because lymph node metastatic NSCLCs were characterized by a significant increase in tumor periphery LVD, we next used Kaplan-Meier analysis to calculate the overall survival rate for patients with low and high tumor periphery LVD. This classification was based on the median values of LVDs in our patient population. We found that a high level of lymphangiogenesis in the peripheral portions of the tumors was a significant prognostic factor for reduced overall survival. The 5-year survival rates of patients with high LYVE-1-LVD and patients with low LYVE-1-LVD were 23.3% and 68.7%, respectively. The 5-year survival rates of patients with high versus low D2-40-LVD were 30% and 67.2%, respectively. However, if we classified the tumors according to their angiogenic phenotype, this tendency proved to be significant only in the angiogenic tumor group, and there was no statistically significant information from the LVDs of non-angiogenic tumors. As non-angiogenic tumor phenotype was found to be related to the risk of lymph node metastasis, we also analyzed the overall survival of our patient population according to the angiogenic phenotype of their tumor, and found that patients with non-angiogenic tumors have a significantly poorer survival than those with angiogenic tumors. The 5-year survival rates of patients with non-angiogenic versus angiogenic tumors were 26.3% and 54.8%, respectively.

Although we failed to show prognostic information from the level of lymphangiogenesis (LVD scores) in non-angiogenic tumors, analysis confirmed that non-angiogenic phenotype is an adverse prognostic factor for overall survival. Multivariate analysis (including standard prognostic parameters such as tumor extension, lymph node status, and patient age) also showed that tumor periphery LVDs predicted outcome independent of other variables. Further prognostic factors related to poor survival were tumor stage and lymph node metastasis. In case of patients with angiogenic tumors a high level of lymphangiogenesis in the peripheral portions of the tumors proved to be an independent unfavorable prognostic factor as well.

We tried to find a correlation between the level of circulating LVEPCs and the survival time of SCLC patients. Because lymphatic involvement of SCLCs was associated with increased LVEPC counts, we next used Kaplan-Meier analysis to calculate the overall survival rates for patients with low and high PB LVEPC levels. We found that patients whose PB samples were categorized by low pretreatment CD34+/VEGFR3+ LVEPC levels had significantly longer survival times than those with high levels of circulating LVEPCs. Multivariate analysis (including standard prognostic variables, such as age, gender, tumor and lymph node stage) also indicated that pretreatment circulating LVEPC levels predicted outcome independent of other variables.

IV/5. Lymphatic proliferation

To explore the proliferation status of the lymphatic network in NSCLC, we carried out double immunostaining with antibodies against D2-40/podoplanin and the proliferation-associated Ki67 nuclear protein. The results corroborated Ki67 nuclear staining in lymph vessel endothelial cells and, as anticipated, in the cancer cells themselves. The endothelial cell labeling index of lymphatic vessels

was always the highest peritumorally and was always lower in the tumor centre than in the periphery. The most extreme situation was that of the non-angiogenic tumor group, where a moderate number of dividing lymphatic endothelial cell nuclei were observed peritumorally, but no Ki67 staining was observed intratumorally. This evidence suggests that the intratumoral lymphatics in non-angiogenic human NSCLCs are indeed preexisting lymphatics that have simply been co-opted and entrapped by the growing tumor mass. In contrast, assessment of the proliferation status of lymphatic endothelial cells of tumors exhibiting an angiogenic growth pattern has shown that lymphatic endothelium can actively divide in all of the investigated areas.

IV/6. VEGF-C expression in human NSCLC

Of 103 cases with NSCLC, 56 cases were positive for VEGF-C. No staining was observed in the normal lung tissue. The staining pattern was either diffuse (mainly in angiogenic growth type), or focal (mainly in non-angiogenic growth type). VEGF-C was significantly more frequently expressed in angiogenic carcinomas when compared with non-angiogenic tumors. However, no significant associations with age, gender, histologic type, tumor differentiation, tumor (T) status, lymph node (N) status, smoking history or disease stage were detected. Furthermore, there was no significant correlation between the intensity of VEGF-C expression and the lymphatic endothelial cell labeling index or LVDs as evaluated by podoplanin and LYVE-1 immunostaining. Accordingly, we were unable to detect the prognostic role of VEGF-C expression in a multivariate analysis.

IV/7. Characterization and levels of LVEPCs in PB samples of SCLC patients

In our study we determined the numbers of CD34+/VEGFR3+ double positive LVEPCs in the PB of 32 control subjects and 88 SCLC patients by flow cytometry. In the control group, the median value of CD34+/VEGFR3+ circulating LVEPCs was 455/mL. In patients with SCLC, this level was significantly higher, with a median value of 1625 /mL.

IV/8. Correlations between LVEPC levels and clinicopathological parameters

LVEPC numbers were also evaluated according to the clinicopathological factors of our patients. There was a statistically significant relationship between LVEPC levels and lymph node involvement. However, no significant associations with age, smoking history, gender or tumor (T) stage were detected.

IV/9. Peripheral blood levels of VEGF-C in SCLC patients

Although VEGF-C serum levels of patients were significantly elevated as compared with those of control subjects, we were unable to detect a significant relationship between the concentrations of the key lymphangiogenic molecule, VEGF-C, and circulating CD34+/VEGFR3+ LVEPC counts. Moreover, when VEGF-C levels were evaluated according to the clinicopathological factors of our patients, no significant associations with age, smoking history, gender, or more interestingly, with lymph node status, tumor stage or survival were detected.

V. Discussion

V/1. The investigation and significance of lymphangiogenesis in patients with NSCLC

In angiogenic NSCLC the lymph vessels of the tumor periphery are more important in the metastatic process than the peritumoral lymph vessel network (corresponding to the "lymphatic hot spots") or the demolished and therefore non-functional lymphatics of the tumor centre.

Although non-angiogenic growth type was associated with a significant risk for the development of lymph node metastasis and to a shorter survival in patients with NSCLC, we failed to show prognostic information from the LVD scores in any investigated part of these tumors. The putative non-angiogenic tumors are highly aggressive, and co-opted lung lymphatics could still play a key role in addition to pre-existing blood capillaries. The normal lymphatics incorporated into the tumor could be more effective than the newly formed, because in non-angiogenic NSCLCs lymph drains directly into the incorporated host lymphatic network, whereas in angiogenic cancers only in peripheral areas is the lymph drained into the lymphatics of the surrounding normal tissue. In that way, lymphatic dissemination of cancer cells may occur via the pre-existing lung lymphatics within the entire mass of non-angiogenic cancers.

Although in various malignant tumors, including NSCLC, VEGF-C expression has been reported to be significantly correlated with lymph node metastasis, and we found elevated VEGF-C immunoreactivity in NSCLC as compared to that in normal lung tissue, there was no obvious association between expression of VEGF-C and LVDs, lymph node metastasis or patients' survival. Moreover, we failed to detect an association between VEGF-C expression and the lymphatic endothelial cell labeling index. It is difficult to conclude, therefore that lymphangiogenesis in human NSCLC is a result of VEGF-C action, as recent studies using experimental tumor models have found. Our results rather suggest that VEGF-C most probably acts as a survival factor in NSCLC. This

idea was further corroborated by the observation that in non-angiogenic tumors with no evidence of intratumoral lymph vessel sprouting, the expression of VEGF-C was restricted to the direct vicinity of co-opted host lymphatics. However, chances are that as in tumor-induced angiogenesis, where the interaction of multiple cytokines controls tumor vascularization, the dynamic balance of several lymphangiogenic growth factors is also likely to determine the activity of lymphangiogenesis.

V/2. The investigation and significance of lymphovasculogenesis (LVEPC-s) in patients with SCLC

This study presents the novel finding that patients with SCLC have PB circulating CD34+/VEGFR3+ LVEPC numbers significantly higher than those in tumor-free control subjects, and that the levels of these cells correlated to lymphatic progression and to clinical behavior. LVEPCs could contribute to both lymph and blood capillary growth of human SCLCs. The data from this current study do not allow us to measure the vasculogenic activity of LVEPCs or determine the ratio of LVEPC contributions between vasculogenesis and lymphvasculogenesis. However, given the observation that LVEPC numbers were related to the extent of lymph node metastases, one can hypothesize a potential role for these cells in the lymphangiogenic machinery, or at least the possibility that the driving force behind the lymphatic progression of SCLC and the mobilization of LVEPCs from the bone marrow is similar.
Based on the above theory, one can assume that our observation on increased LVEPC numbers is the result of elevated levels of the VEGFR3 ligand VEGF-C. Thus, we assayed the PB levels of VEGF-C and found that although its concentrations were significantly higher in SCLC patients than in control subjects, no statistically significant relationship existed between VEGF-C levels and numbers

of circulating LVEPCs. In addition to the observation of significantly higher pre-treatment circulating LVEPC counts in SCLC patients as compared with control subjects, this prospective study presents the novel finding that a single flow cytometric measurement of CD34+/VEGFR3+ LVEPCs is a useful tool to predict outcomes in patients with SCLC. During the follow-up period of 25 months, a significantly higher incidence of death from SCLC was observed in patients with high pre-treatment LVEPC levels as compared with patients with low LVEPC levels, suggesting that the pre-treatment levels of LVEPCs, detectable by flow cytometry in the PB, correlate with the clinical behavior of human SCLC.

However, although we demonstrated elevated VEGF-C concentrations in SCLC patients over tumor-free controls, we failed to detect an association between VEGF-C levels and patients' survival, and analysis of the cancer patient cohort showed no differences between clinicopathological subgroups. In particular, no difference in the VEGF-C levels was seen between patients with N0-1 and with N2-3 stages.

VI. Conclusions

In conclusion, NSCLC metastasis to lymph nodes is a key event in disease outcome, and is frequently used as a prognostic factor. Our study demonstrates, for the first time, that lymphangiogenesis occurs exclusively in the angiogenic growth type of human NSCLCs, and that LVD is correlated to clinical behavior and to lymph node status only in this growth type of NSCLCs. However, it also provides the first evidence that the risk of lymph node metastasis as well as a shorter survival was more likely to occur in the patient population with non-angiogenic tumors, and that these tumors mainly co-opt host tissue lymphatics during their growth, in contrast to most of the angiogenic ones, which expand with concomitant

lymphangiogenesis. The latter suggests that the co-opted host lymphatics of human NSCLC are more important in the metastatic process than the newly formed ones. This assumption, however, would need further experimental and clinical support.

The current study demonstrates, for the first time, that the circulating numbers of bone marrow–derived LVEPCs are significantly increased in SCLC patients and that these numbers correlate with the extent of tumor spread to regional lymph nodes and with patients' survival. Although our data suggest a participation of LVEPCs in lymphatic tumor progression in SCLC patients, it is not clear yet whether LVEPCs play a role only in the lymphatic spread of the tumor, or whether they also facilitate primary tumor growth and the development of blood-borne metastases via the enhancement of blood capillarization. Moreover, it has yet to be determined if LVEPCs can be used as a surrogate marker to monitor the efficacy of standard or future anti(lymph)angiogenic therapies in SCLC. Further research is also needed on whether LVEPCs can be targeted to treat patients with SCLC, or alternatively—as they are endowed with the capacity to home to the tumor lymphatic network— can be manipulated to deliver toxins or lymph vessel-targeting agents. Finally, because the above results are most likely not specific for SCLCs, they may lead to a number of novel approaches in the diagnosis and treatment of other malignant diseases as well.

Publications related to the thesis

1. High VEGFR-3-positive circulating lymphatic/vascular endothelial progenitor cell level is associated with poor prognosis in human small cell lung cancer

Bogos K, **Renyi-Vamos F**, Dobos J, Kenessey I, Tovari J, Timar J, Strausz J, Ostoros G, Klepetko W, Ankersmit HJ, Lang G, Hoda MA, Nierlich P, Dome B.

Clin Cancer Res. 2009 Mar 1;15(5):1741-6. Epub 2009 Feb 24. IF:6.25

2. Circulating endothelial cells, bone marrow-derived endothelial progenitor cells and proangiogenic hematopoietic cells in cancer: From biology to therapy

Dome B, Timar J, Ladanyi A, Paku S, **Renyi-Vamos F**, Klepetko W, Lang G, Dome P, Bogos K, Tovari J.

Crit Rev Oncol Hematol. 2009 Feb;69(2):108-24. Epub 2008 Sep 3.IF: 4.632

3. Lymphangiogenesis correlates with lymph node metastasis, prognosis, and angiogenic phenotype in human non-small cell lung cancer

Renyi-Vamos F, Tovari J, Fillinger J, Timar J, Paku S, Kenessey I, Ostoros G, Agocs L, Soltesz I, Dome B.

Clin Cancer Res. 2005 Oct 15;11(20):7344-53. IF:5.715

Publications unrelated to the thesis

4. Role of retinoic receptors in lung carcinogenesis

Bogos K, **Renyi-Vamos F**, Kovacs G, Tovari J, Dome B.

J Exp Clin Cancer Res. 2008 Jul 14;27:18. IF:1.5

5. Giant cystic pheochromocytoma located in the renal hilus. Melegh Z, **Rényi-Vámos F**, Tanyay Z, Köves I, Orosz Z. Pathol Res Pract. 2002;198(2):103-6; discussion 107-8. IF:0.85

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