THE ROLE OF WNT SIGNALING IN THE DEVELOPMENT AND TREATMENT OF NON-SMALL CELL LUNG CANCER

Ph.D. Thesis

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INTRODUCTION

Non-small cell lung cancer

Lung cancer (LC) with disappointing survival statistics represents the second most common forms of cancers in both men and women worldwide. The two main types of LC-s are small cell lung cancer (SCC) and non-small cell lung cancer (NSCLC) where the latter can be further classified into adeno (AC)-, squamous cell (SCC) - large cell (LCC) and various mixed types carcinomas accounting all together for approximately 85% of all LC cases. As the majority of patients are diagnosed at an advanced stage of the disease, the outcome is poor and the overall 5-year survival rarely exceeds 15%. Naturally, earlier recognition would improve the outcome, but currently only a few treatment options are available to lung cancer sufferers that are largely based on identified driver mutations. Unfortunately, only a small percentage of NSCLC patients have such characteristic mutations therefore the majority cannot benefit from targeted therapy.

NSCLC cases can be characterized with KRAS, EGFR mutations and ALK rearrangement, but those are not found in squamous cell carcinoma patients. Activating EGFR mutations usually occur within exon 18 and 21, which result in enhanced sensitivity for EGFR small molecule tyrosine kinases, such as gefitinib and erlotinib. Although KRAS mutation frequency is the highest in the Caucasian population, no treatment option is available yet in KRAS positive lung cancer patients.

Anti-angiogenic therapy in lung cancer

VEGF-A has been identified as a key regulator of both normal and pathological angiogenesis. In normal tissue during dormancy whereby angiogenesis is inhibited, levels of inhibitors and activators are equal, but alteration of pro-angiogenic or anti-angiogenic balance can facilitate tumor angiogenesis. This phenomenon is called “angiogenic switch” which favours abnormal angiogenesis. So the tumor can induce new blood vessel formation, but the newly formed network is often leaky, poorly differentiated and not hierarchic. Recognition that new blood vessel formation is important for tumor growth allowed the clinical application of anti-angiogenic approach. The idea of anti-angiogenic therapy came from Judah Folkman from the early 70s.

The first monoclonal antibody –bevacizumab- was approved against human VEGF-A the key regulator of angiogenesis. As VEGF-A promotes endothelial cell survival, migration, proliferation and vascular permeability it appears an ideal target to “starve” the tumor and lead to tumor regression. High VEGF-A expression in NSCLC tumors and its contribution in tumor progression makes it an appropriate candidate for therapy.

Despite some success of bevacizumab mostly in combination therapy, patients mainly with squamous histology were excluded from treatment as increased risk of fatal side effects were observed. The reasons for serious haemorrhage are still unknown, but several ideas have come to light. For example, the two types of NSCLC-s are not only differ in genomic mutations, but AC and SCC possess different intratumoral blood vessel formations also and intratumoral vessels are less covered by pericytes in SCC than AC, leading to more vulnerable and fragile

vascular wall with increased necrosis in newly formed vessels in SCC. Solely blocking VEGF-A simply cannot provide an overall therapeutic solution in NSCLCs, as alternative signaling pathways also play a significant role in the regulation of angiogenesis.

Role of PPARgamma in carcinogenesis

PPAR molecules are transcription factors of a nuclear hormone receptor superfamily. PPARs are important regulators of lipid storage and metabolism, but PPARs have been directly linked to tumorigenesis. As PPARgamma ligands can inhibit tumor cell proliferation, the involvement of PPARgamma ligands have been extensively studied regarding their potential antitumor capacity. PPARgamma activation by an agonist ligand can inhibit tumor growth, although the colon tumor size in APC mutant mice have been increased. One of the controversial regulators of VEGF-A is also the PPARgamma that has been reported to inhibit endothelial cell function and vasodilatation. According to the growing literature, PPARgamma can either activate or inhibit VEGF-A mediated endothelial cell response depending on the modulatory effect of the surrounding molecular microenvironment that expresses various additional regulators of angiogenesis.

Wnt5a and angiogenesis

The Wnt family of secreted glyco-lipo-proteins control a wide variety of cellular processes including cell fate specification, cell proliferation, cell polarity and cell migration therefore important in both fetal development and carcinogenesis. Their role in lung carcinogenesis has been also described, as enhanced activation of Wnt /beta-catenin pathway in Kras mutant mice lead to a more aggressive phenotype of the tumor. The Wnt signaling pathway, however, is not a single pathway. The most characterized Wnt pathway is the beta-catenin dependent canonical pathway. The Wnt signaling can also function in beta-catenin independent manner, called non-canonical Wnt signaling, including the Ca^{2+} and the planar cell polarity (PCP) pathways. Wnt-ligands, especially the non-canonical Wnt5a are also important regulators of endothelial cell division, survival and migration, consequently angiogenesis has also been proposed to be under Wnt control. Wnt5a expression has been investigated in 205 NSCLC samples and immunohistochemical analysis has revealed that Wnt5a expression significantly correlates with vascular mimicry. As canonical and non-canonical Wnt pathways are differentially active in AC and SCC, we considered the possibility that differences in the Wnt microenvironment can be partly responsible for variations in the tumor angiogenesis. Wnt5a was specifically selected as its up-regulation is characteristic to SCC tumors and its upregulation might also be responsible for alterations in blood vessel formation leading to serious side effects of anti-angiogenic therapies.

AIMS OF THE STUDY

1. *How VEGF-A changes during aging and lung carcinogenesis and what is its role in the altered angiogenesis?*

2. *Can canonical or non-canonical Wnt microenvironment affect PPARgamma expression? What molecules are responsible for the altered regulation?*

3. *What are the functional consequences of altered Wnt microenvironment regarding endothelial cells? How Wnt molecules influence the angiogenesis of AC and SCC?*

MATERIALS AND METHODS

Cell lines and primary cells

Human foreskin fibroblast cell line (F11, System bioscience Mountain View, CA, USA) and human lung adenocarcinoma A549 (American Type Culture Collection, Rockville, MD) cell line were used for the experiments. VEGF overexpressing F11 cell line was generated in our laboratory. Normal primary human small airway epithelial cells (SAEC), normal human lung fibroblast (NHLF) and human microvascular lung endothelial cells (HMVEC-L) were purchased from Lonza, isolated from anonymous donors of different ages and sex.

3D in vitro lung tissue aggregates

To create a fully human 3D lung tissue model, SAEC, NHLF and HMVEC-L cells were used. All cells were cultured at 37°C and 5% CO_2 in primary cell culturing media. After the cells reached 80% confluence, all types were sub-cultured and mixed [30% SAEC, 30% HMVEC-L and 40% NHLF together and dispensed onto a low-attachment 96-well U-bottom plate (Corning). Cells were centrifuged at 600g for 10 minutes and maintained at 37°C and 5% CO_2 in mixed SAGM:EGM-2:FGM-2 media during the experiments. Throughout the experiments, aggregates were treated with 10 mM LiCl (Sigma-Aldrich) for 48h and recombinant human Wnt5a and Wnt11 (R&D Systems) for 72h.

Human samples

Lung tissue samples (Supplementary table 1) were collected during lung resections at the Department of Surgery, University of Pécs, Hungary. The project was approved by the Ethical Committee of the University of Pécs. Patients had given written consent to provide samples for research purposes. All collected samples were treated anonymously.

Animals

For the experiments C57BL/6 and PPARgamma knock-out mice were used from both genders. Mice were kept under standardized conditions, where tap water and food was provided ad libitum. Animals were sacrificed at the age of 3.5 months.

Gene expression studies

Total RNA from cell cultures was extracted with MN NucleoSpin RNA isolation kit according to manufacturer's protocol (Macherey-Nagel). The concentration of RNA samples was measured using NanoDrop (Thermo Scientific).

Total RNA from human lung tissues were obtained using TRIzol reagent (Invitrogen). 1 μg RNA were digested with DNase (Sigma-Aldrich). cDNA was synthesized with high capacity RNA to cDNA kit (Life Technologies) using 1 μg of total RNA according to manufacturer's recommendation. RT-PCR was performed using SensiFAST SYBR Green reagent (BioLine,

London, UK), Taqman Wnt array plate and Taqman microRNA assay. Amplifications were run on ABI StepOnePlus system. Gene expression were analyzed with StepOne software.

Immunofluroescent and hematoxylin-eosin staining

Mice were anaesthetized with sodium pentobarbital intraperitoneally and lungs were filled up with 1:1 ratio of PBS:cryostate embedding media (TissueTek Alphen aan den Rijn, Netherland), and frozen down at -80°C. The human samples were collected in PBS containing 1% of FBS and then were filled up with PBS:cryostate embedding media and kept at -80°C until processing. The 3D lung aggregates were carefully removed from the 96- well plates and embedded into TissueTek embedding media and immediately frozen down at -80°C. For histological observations, 8 µm thick cryostat sections were fixed with 4% PFA for 20 minutes.

Fixed tissues sections were rehydrated and blocked for 20 minutes in 5% BSA in PBS. Primary antibodies were applied for 1 hour. The secondary antibodies were Alexa Fluor 488 or 555 conjugated anti-mouse IgG antibodies (Life Technologies). The nuclei were counterstained with TO-PRO-3 (Life Technologies) and showed in blue as pseudo-color blue. Pictures were captured using Zeiss LSM 710 microscope equipped with analysis software. Images and fluorescent intensity were measured with Fiji software. Intensity of two groups was analyzed with the Student t test. 8 µm thick cryostat sections or Transwell inserts (Corning, New York, USA) were cut and stained in Mayer's hematoxylin solution (Sigma-Aldrich, St. Louis, USA) for 10 minutes. Sections were washed in running tap water for 10 minutes, then differentiated with 0.25% acetic acid (Sigma Aldrich, St. Louis, USA) for 1 minute. After the differentiation step, slides were washed with tap water and stained in eosin solution for 2 minutes, then washed. Sections were mounted using Vectashield mounting medium (Vector Laboratories, Burlingame, USA). Images were taken using Nikon Eclipse Ti-U inverted microscope (Tokyo, Japan).

PPRE reporter assay

A549 cells were transfected with PPRE-luciferase reporter and PPRE control-luciferase reporter vectors using Lipofectamine 3000 (Thermo Fisher Scientific, Waltham, USA). 6*103 cells were transfected with 100 ng of plasmid DNA mixed with 0.3 µl Lipofectamine 3000 and 0.2 µl P3000 reagent (Thermo Fisher Scientific, Waltham, USA). After overnight incubation, cells were treated with LiCl at 10mM concentration for 24hours. Luciferase reporter, containing PPRE responsive element can be activated by PPAR activation, which catalyzes luciferin oxyluciferin transformation into luminescent signal. PPAR activation was measured using BrightGlo luciferase assay and detected by BioTek Synergy HT plate reader. Changes in PPAR activation were compared to PPRE control plasmid. RNA isolation was performed after 24hours treatment. Gene expression was compared to non-treated cell cultures from three independent experiments. For protein detection, transfected cells were treated with LiCl at 10 mM concentration for 72 hours. At the endpoint of the treatment; protein transport was blocked with 5mg/ml Brefeldin-A for 4 hours, as VEGF-A is a soluble factor. Immunfluorescent staining was performed using purified primary anti-human VEGF-A antibody, visualized by Alexa Fluor 488 conjugated anti-mouse secondary IgG antibody. Nuclei were stained by TO-PRO3 and pseudo-colored for blue. Pictures were taken by Zeiss LSM 710. Images are representative from three different experiments.

PPARgamma agonist and antagonist treatment

F11 cell line was cultured in 24-well plate and was treated with 10 μM rosiglitazone (RSG) and 10 μM GW9662 (Sigma-Aldrich, St. Louis, USA) for 48h in the presence or absence of rhWnt5a (R&D Systems, Minneapolis, USA). VEGF-A mRNA level was determined using real-time quantitative PCR, while miR-27b expression was measured by Taqman MicroRNA Assay (Thermo Fisher Scientific, Waltham, USA).

HMVEC-L Transwell migration assay

HMVEC-L cells were seeded onto the Transwell insert (8 μm pore size, 6.5 mm diameter) (Corning Costar, Sigma Aldrich, St. Louis, USA) at the density of 2*104. To assess the effect of VEGF-A and Wnt5a, HMVEC-L were cultured in the presence or absence of elevated level of VEGF-A and recombinant human Wnt5a. To gain VEGF-A excess, F11-VEGFhigh cells were seeded into the well. F11-VEGFnormal cells were used as controls. After 24h, the inserts were stained with Hematoxylin-eosin (detailed protocol below) and pictures were taken using Nikon Eclipse Ti-U inverted microscope (Tokyo, Japan).

Flow cytometry

3D SAEC-F11 $VEGF^{high}$- HMVEC-L and SAEC-F11-HMVEC-L lung aggregates were cultured for 72hours in the presence or absence of rhWnt5a. Aggregates were then dissociated with Accumax™ (Sigma-Aldrich) solution and washed in PBS once. Single cell suspensions were incubated with Allophycocyanin (APC) conjugated anti-human CD105 (Clone 43A3, BioLegend) and Brilliant Violet 421 conjugated anti-human CD31 (Clone VM59, BioLegend) for 30 minutes at room temperature in dark. Cells were washed in PBS, fixed with 1% PFA and stored at 4°C in dark until FACS analysis. Labeled cells were analyzed with FACS Canto II flow cytometer (BD Immunocytometry Systems, Erembodegen, Belgium) with BD FACS DIVA software V6 and data were analyzed with FCS Express V3 software.

Statistical analysis

Statistical analysis was performed with SPSS version 20 software. Data are presented as mean ± standard error of mean (SEM), and statistical analysis was performed using the independent sample t-test and one-way ANOVA with Bonferroni correction. $p<0.05$ was considered as significant.

RESULTS

VEGF-A expression in the lung

Significantly increased VEGF-A expression was detected in old lung samples compared to young as controls as well as in both AC and SCC. PPARgamma levels were reduced in both tumor subtypes compared to normal, non-diseased lung controls. Quantitative analysis highlighted an existing difference in the two NSCLC subtypes, while lower PPARgamma mRNA levels characterized significantly higher VEGF-A expression in AC, higher PPARgamma and lower VEGF-A mRNA described SCC.

Wnt signaling in NSCLC

As tumor progression has been associated with Wnt molecules, we studied canonical and non-canonical Wnt signaling of AC and SCC samples. Elevated mRNA and protein level of non-canonical Wnt5a was associated with SCC samples in contrast to AC patients.

Connection between the canonical Wnt signaling and PPARgamma

Investigating the role of Wnt and PPAR signaling pathway in VEGF-A production we studied the association between PPARgamma and Wnt pathway activation. PPRE plasmids containing specific PPAR binding site were used to determine PPAR activity. Treatment of A549 AC cell line with beta-catenin activator LiCl resulted in decreased promoter activity, while IWR-1 had no significant effect. Interestingly, VEGF-A mRNA level increased by both treatment, but LiCl led to increased protein level, while IWR-1 caused no significant change of VEGF-A protein expression. Wnt5a staining of the lungs of PPARgamma knock-out mice highlighted the importance of signaling activity, as the absence of PPARgamma did not lead to change in Wnt5a protein level demonstrating that Wnt5a is not under PPARgamma control.

Wnt5a induces miR-27b a regulator of PPARgamma expression

Based on the literature and experiments with the canonical Wnt pathway it appeared that only beta-catenin dependent signaling pathway activation is able to reduce PPARgamma expression and increase VEGF-A levels. It was not clear how SCC samples with high levels of the non-canonical Wnt5a can trigger the same mechanism. miRNAs have been reported as regulators of angiogenesis and both miR-27b as well as miR-200b have been connected to Wnt, PPARgamma and VEGF-A pathways therefore we theorized that differences in miRNAs might explain the observed variations in blood vessel network formations of lung AC and SCC. To investigate, both miR-27b and miR-200b levels were measured in primary human AC and SCC samples. Significantly higher expression levels were determined for both miRNA in primary SCC tissues compared to AC. To test whether the different molecular microenvironment characterized by increased Wnt5a levels in SCC would explain variation in miRNA expression, 3D lung aggregate cultures were exposed to non-canonical rhWnt5a. miR-27b expression was significantly increased by rhWnt5a treatment of the test tissue. In contrast, miR-200b levels were unaffected indicating a previously unidentified regulatory mechanism amongst signaling pathways of angiogenesis regulation. PPARgamma agonist and antagonist treatment along with rhWnt5a revealed that RSG can inhibit Wnt5a induced miR-27b increase, while antagonist GW9662 did not show the same effect. Interestingly, decreased VEGF-A expression level was detected in PPARgamma antagonist and Wnt5a combination treatment compared to antagonist alone. Such treatments led to the same patterns as in SCC cases.

The role of Wnt5a in endothelial cell proliferation

To be able to study the molecular regulation of VEGF-A induced endothelial cell functions, in vitro studies were performed using a three dimensional (3D) human lung aggregate model consisting of primary human small airway epithelial cells (SAEC), microvascular lung endothelial cells (HMVEC-L), and human fibroblasts (F). The 3D aggregate culture conditions

provided close to natural, yet defined, cellular environment for molecular studies. qRT-PCR analysis of tissue aggregates revealed highly similar expression levels of angiogenic stimulators including VEGF-A, IL-1beta and HIF-1alfa to primary human lungs indicating that the model is suitable to study pro- and/or anti-angiogenic mechanisms. As VEGF-A has previously been determined to stimulate endothelial cell functions, expression of CD105 marker, a transmembrane auxillary receptor for transforming growth factor-beta (TGF-beta) that is predominantly expressed on proliferating endothelial cells was also tested. The analysis of primary SCC and AC tissue samples showed significantly lower expression in SCC compared to AC tissue samples. Significantly lower PPARgamma and higher VEGF-A expression in both tumor tissue types is expected to generate enhanced angiogenesis, but analysis of CD105 showed a reverse pattern. Contrary to our expectations decreased CD105 protein level was detected in CD31 positive endothelial cells of lungs in PPARgamma KO mice which is a similar pattern detected in human lung SCC samples. Based on the above results we hypothesized that elevated levels of VEGF-A alone are not sufficient to induce endothelial proliferation. To test whether rhWnt5a has a modulatory effect on pro-angiogenic mechanisms in a tumor-like VEGF-A^{high} environment, aggregates containing VEGF-A^{low} and VEGF-A^{high} fibroblasts were studied in the presence or absence of rhWnt5a. Flow cytometric analysis revealed that added rhWnt5a cannot block the expression of the endothelial cell proliferation marker CD105, indicating that CD105 marker is not under Wnt5a control.

Cadherins

Changes in the balance of different cadherin subtypes are a trademark of tumorigenesis. Treatment of recombinant Wnt5a resulted in decreased E-cadherin and increased N-cadherin mRNA levels. Investigating endothelial VE-cadherin in 3D lung aggregates we found that VEGF-A excess enhanced both N- and VE-cadherin, and that rhWnt5a had an additive effect and increased both mRNA levels even further.

Wnt5a inhibits endothelial cell migration

Our previous results indicate that Wnt5a is not a direct regulator of pro-angiogenic factor transcription, but it can alter VEGF-A mediated processes. To test whether rhWnt5a has a modulatory effect on pro-angiogenic mechanisms in a tumor-like VEGF-A^{high} environment, aggregates containing VEGF-A^{low} and VEGF-A^{high} fibroblasts were studied in the presence or absence of rhWnt5a. To detect the localization of endothelial cells in culture, tissue sections were prepared and stained for the CD31 endothelial cell marker. While in the VEGF-A^{low} microenvironment endothelial cells were evenly distributed in the tissue, in the presence of VEGF-A^{high} fibroblasts endothelial cells migrated towards the VEGF-A signal of fibroblasts - that naturally provide the core of the 3D lung tissue aggregate co-culture- and formed a readily detectable endothelial cell stream. Added rhWnt5a inhibited endothelial cell migration towards the VEGF-A^{high} fibroblasts and the endothelial cells showed the same distribution pattern that was observed in the VEGF-A^{low} microenvironment. Transwell migration of HMVEC-L cells confirmed the regulatory role of Wnt5a. While endothelial cells migrated faster towards VEGF-A^{high} microenvironment, added rWnt5a inhibited VEGF-A mediated migration.

SUMMARY

1. *How VEGF-A changes during aging and lung carcinogenesis and what is its role in the altered angiogenesis?*

VEGF-A mRNA level in aging lung is increased and the endothelial proliferation marker CD105 is also elevated. As NSCLC incidence is higher in the elderly it was not so surprising that the molecular pattern is similar in both NSCLC subtypes to lung samples of aging people. We have also reported that decreased PPARgamma expression is connected to VEGF-A increase. Our in vitro lung aggregates proved to be a suitable model system to investigate VEGF-A effects where we have shown that VEGF-A excess enhance endothelial proliferation and migration in 3D tissue aggregates.

2. *Can canonical or non-canonical Wnt microenvironment affect PPARgamma expression? What molecules are responsible for the altered regulation?*

Significantly reduced PPARgamma and increased VEGF-A levels in both AC and SCC samples were detected which result indicates increased angiogenesis. Different therapeutic response could not be explained by simply these two molecules. In our study, we described that PPARgamma mRNA and protein level can be reduced by activation of beta-catenin dependent Wnt pathway. Activation of canonical Wnt pathway also led to induced VEGF-A production via PPARgamma decrease. As the canonical Wnt pathway is more active in AC than SCC the above findings explained such an increase in VEGF-A in lung AC samples. The similarly high VEGF-A and reduced PPARgamma levels in SCC samples seem counter-intuitive as in SCC the non-canonical, beta-catenin independent Wnts play the dominant role. In non-canonical microenvironment, the inhibition of PPARgamma could be explained by Wnt5a induced upregulation of miR-27b that can inhibit PPARgamma.

3. *What are the functional consequences of altered Wnt microenvironment regarding endothelial cells? How Wnt molecules influence the angiogenesis of AC and SCC?*

Further investigation of Wnt5a revealed that Wnt5a cannot inhibit VEGF-A induced endothelial cell proliferation. Nevertheless, expression of cadherins that are important molecules in maintenance of cell-cell interaction and tissue integrity, changed after rhWnt5a treatment. Recombinant Wnt5a treatment of primary epithelial cell culture led to cadherin switch including E-cadherin decrease and N-cadherin increase. Using 3D lung aggregates as model systems we detected increased N- and endothelial VE-cadherin mRNA levels in the presence of Wnt5a. Functional tests also proved the inhibitory effect of Wnt5a on endothelial cell motility. Primary endothelial cells migrated faster in the presence of VEGF-A^{high} envorinment, while Wnt5a excess inhibited endothelial migration. These facts highlight the importance of the cancer type specific microenvironment on endothelial cells and partly explain the more fragile vascular wall in SCC making SCC patient more prone to haemorrhage upon anti-vascularization treatment.

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Cumulative impact factor: **10.55**
Cumulative impact factor of articles related to the thesis: **5.032**

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