Preclinical examination of therapeutic targets in thoracic tumors

Doctoral theses

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INTRODUCTION

Lung cancer means an important medical problem globally, it accounts for 13% of the total cancer cases and 18% of the cancer deaths in 2008.About 15% of all lung cancer cases are small cell lung cancer (SCLC), the major proportion, 85% are non-small cell lung cancer. This group can be divided into 3 main subtypes: the squamous cell carcinoma (SCC), the adenocarcinoma (ADC) and the large cell carcinoma (LCC).

The other group of thoracic tumors, the malignant pleural mesothelioma (MPM) is a highly lethal malignancy. It affects the serosal lining of the pleural cavity and is thought to develop from superficial mesothelial cells. MPM is strongly associated with asbestos exposure and shows a 20-40 years latency period. The expected median survival of patients suffering from MPM is still poor ranging from 4 and 12 months from diagnosis.

Treatments with targeted molecular drugs have recently offered novel therapeutic strategies in the case of cancer, including thoracic tumors. The understanding of molecular mechanisms that underlie tumor growth and angiogenesis is essential to find appropriate molecular targets. Further efforts are therefore needed for the better knowledge of several angiogenic, lymphangiogenic molecules and oncogene proteins.

In this study we examined the potential role of the recently discovered angiogenic peptide, the apelin molecule in angiogenesis of NSCLC, and in the lympangiogenesis.

The receptor of apelin was identified in 1993, the ligand was first isolated from bovine stomach homogenates in 1998.

Several studies were published recently the role of apelin molecule in angiogenesis. During embrionic development, APJ expression is largely restricted to the endothelial and endothelial progenitor cells of the forming heart and the primary blood vessels. Apelin is essential for regular vascular patterning of the frog embryo. Apelin and its receptor are also highly expressed in the adult vessel walls, especially in blood endothelial cells.

Apelin was reported to stimulate the *in vitro* growth of human umbilical and mouse brain microvasculature-derived endothelial cells and the *in vitro* migration and capillary-like tube formation of monkey retinal endothelial cells. In line with that, apelin was reported to stimulate *in vivo* angiogenesis in the chicken chorioallantoic membrane and in the mouse subcutaneous matrigel plug assay systems. The apelin/APJ system is involved inthe regulation of blood vessel diameter during angiogenesis.

The increased expression of apelin is observable in some cancer types. The APJ receptor and its ligand is highly expressed inthe angiogenic tumor vasculature inthe case ofglioblastomamultiforme. Apelin also expressed the malignant tumor cells of invasive or lobular breast carcinoma. The highly expression of apelin increased the neoangiogenesis and the *in vivo* growth of murine breast cancer and melanoma. It was published recently that apelin is expressed in oral squamous cell carcinoma, and it is a new prognostic factor in this cancer type. The APJ receptor is also highly expressed by the endothelial cells of colon26 murine and Lewis lung adenocarcinoma.

Blood vessel enlargement by apelin was also observed in tumors developed from PC3 human prostate cancer cells and B16 mouse melanoma cells. We firs studied the role of apelinerg system in NSCLC.

It was published recently that APJ is expressed in human lymphatic endothelial cells, and apelin induced the migration and cord formation of these cells *in vitro*. Furthermore, apelin stabilized the lympahtic endothelial cells in permeability assays. Moreover, activation of apelin/APJ system inhibited the UVB induced inflammation by the inhibition of enlargement of lympahtic and blood vessels *in vivo*. These results indicate that apelin can play a role in the stabilization of lymphatic vessels in inflamed tissues. So we need to clarify the exact role of apelin in physiological as well asin pathological lymph vessel growth using the appropriate *in vitro* and *in vivo* models.

The oncogene protein, the mTOR molecule has been shown to be inhibited by the small molecule, the temsirolimus. Furthermore we investigated the potential of mTOR inhibition by temsirolimus in

malignant pleural mesothelioma.Several studies suggested hyperactivation of the PI3K/AKT/mTOR signal pathway in mesothelioma cells. The mTOR mediated signals support chemotherapy resistance and its blockade exerts chemosensitizing activity in several cancer types. Rapamycin and its analogs such as temsirolimus and everolimus are the best-investigated mTOR inhibitors up to this day. These small molecules have been shown to exert antitumor activity against selected cancers *in vitro* and *in vivo*. Temsirolimus and/or everolimus have been recently approved for the treatment of renal cell carcinoma and mantle cell lymphoma. The mTOR inhibition was studied by NSCLC and MPM, but this area requires further examinations.

AIMS OF THIS STUDY

1. Examination of the expression of apelin and its receptor in NSCLC.
2. Analysis of the *in vitro* effects of apelin on NSCLC.
3. Analysis of the *in vivo* effects of the enhanced apelin expression on NSCLC.
4. Comparative/comprehensive examination of the apelin expression, vascularization and clinical behavior of human NSCLC tumor samples.
5. Examination of the expression of APJ receptor on human lymphatic endothelial cell line.
6. Studies on the *in vitro* effects of apelin on human lymphatic endothelial cells.
7. Characterization of the spheroid formation capacity of the mesothelioma cell lines and the effect of mTOR inhibition on the spheroid formation.

METHODS

Patients

For immunohistochemical analysis, a total of 94 patients with NSCLC treated were selected. None of the patients were treated with neoadjuvant chemotherapy. Histological diagnosis and N stage were determined on hematoxylin and eosin-stained sections. For PCR analysis, fresh surgical tumor and normal lung specimens of 46 NSCLC patients were used.

Cell lines

Human NSCLC and mesothelioma cell lines were used for our experiments. The human lymphatic endothelial cells were foreskin-derived. Foreskins were treated with dispase, followed by mechanically scraping endothelial cells with a cell scraper. The lymaphatic endothelial cells were separated by magnetic sorting and by sorting with an anti-podoplanin-serum. NSCLC cells were transfected with a control or an apelin-encoding pcDNA3.1 vector to develop stable transfectant cell lines.

Drugs

In our *in vitro* experiments we examined the effects of apelin-13, apelin-36, a modified apelin derivative, theF13A and temsirolimus.

Methods of antigen exhibiting

Expression of apelin and its receptor were analysed by immunocytochemical staining on human NSCLC cell lines and human lymphatic endothelial cells. Cellular apelin secretion was measured by ELISA in NSCLC cell lines. Expression of apelin protein and the microvessel density was determined by immunohistochemical staining in human and xenograft tumors. The expression of apelin and its receptor was measured at mRNA level by reverse-transcriptase PCR.

In vitro studies

The proliferation of NSCLC cell lines was measured by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay colorimetrically. The proliferation of human lymphatic endothelial cells was studied by BrdU (5-bromo-2´-deoxyuridine) analysis. The spheoid formation capacity of the cells was studied in ultra low attachment plate, in DMEM/Ham's F-12 medium supplemented with bFGF, EGF and B27. The migration ability of human lymphatic endothelial cells was analysed by videomicroscopy measurements.
The tube formation of the cells was examined on Matrigel, in serum-free medium. Apoptosis assay was carried out using the „in situ cell death detectation" kit. Sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis was carried out for western blot analysis.

In vivo experiments

Growth of the apelin-transfected human NSCLC cells wre compared with those of control vector-expressing cells in xenograft tumors formed in nude mice.

Statistical analysis

All statistical analysis were done using Statistica 8.0 software program.

RESULTS

Apelinand its receptor expression in human NSCLC cell lines

RT-PCR demonstrated the presence of apelin mRNA in the NSCLC cell lines. Immunocytochemical staining showed positive immunoreactivity in the cytoplasm in the case of both apelin and APJ in all of the cell lines. An ELISA for apelin indicated that the cell lines secreted the protein into their cell culture medium.

Comparison of apelin and APJ expression in 2-dimensional and 3-dimensional cultures of NSCLC cell lines

Next, we established 3-dimensional spheroid from 8 NSCLC cell lines. We compared the relative expression levels of apelin or APJ in 2-dimensional and 3-dimensional cultures of the cell lines. The relative apelin or APJ expression level in the 3-dimensional cultures was multiple of the level in the 2-dimensional cultures in the case of apelin in 7 cell lines of the 8, in the case of APJ in 5 cell lines of 8.

Apelin mRNA and protein expression in tumor and normal lung tissue samples of NSCLC patients

Quantitative RT-PCR analysis of 46 paired nontumor and tumor mRNA extracts revealed that apelin levels were significantly higher in the tumor samples of patients compared with the levels of their normal lung tissue specimens.

Immunohistochemical staining of 94 paraffin-embedded NSCLC samples exhibited a cytoplasmic pattern of apelin expression. Apelin protein was expressed by the human bronchial glands and epithelium adjacent to tumors.

Moreover, when apelin mRNA levels were compared with expression levels detected by immunohistochemistry, a significant correlation was found.

Effect of exogenous apelin and apelin overexpression on *in vitro* NSCLC growth

Neither exogenous apelin treatment nor transfection with apelin encoding vector stimulated the *in vitro* proliferation of NSCLC cells, although transfection with apelin encoding vector resulted in a significant elevation in secreted levels of apelin.

Effect of apelin overexpression on *in vivo* NSCLC growth

Tumor growth was significantly accelerated in mice injected with apelin-overexpressing cells compared with mice carrying cells transfected with the empty control vector. Morphometrical analysis revealed a significantly higher microvessel density in the apelin-overexpressing tumors compared with controls. We found the same result in the case of the tumor capillary perimeters.

Comparative examination of the apelin expression, vascularization and clinical behavior of human NSCLC tumor samples

We performed comparative statistical analysis of apelin expression and clinicopathological variables in the case of 94 patients with NSCLC treated. No significant association with age, smoking, gender, tumor (T) status, lymph node (N) status or histologic type were detected. However, high apelin expression was found significantly more frequently in human NSCLC samples with high microvessel density with tumors with low microvessel density. Accordingly, the mean number of blood capillaries counted in high apelin-expressing tumors was significantly greater than that in tumors with low apelin protein expression. Although the tendency toward an increased microvessel perimeter in the cases with high apelin expression was observed, this difference in blood capillary perimeters between tumors with high and low apelin protein expression remained statistically insignificant.

Prognostic significance of the increased expression of the apelin protein

We found that patients whose tumor samples were categorized by low apelin expression had significantly longer survival times than those with high apelin expression. The multivariate analysis of the standard prognostic parameters (such as tumor extension, lymph node status, and patient age) also showed that apelin expressions predicted outcome independent of other variables.

APJ receptor expression in human lymphatic endothelial cells

RT-PCR demonstrated the presence of APJ mRNA in the lymphatic endothelial cells. Immunocytochemical staining exhibited a cytoplasmic pattern of APJ expression.

Effect of exogenous apelin on the human lymphatic endothelial cells *in vitro*

Exogenous apelin treatment did not influence significantly the proliferation of the cells, but it significantly increased the number of spheroids at 10^{-6}M concentration in 3-dimensional culture of the cells. Although it did not influence the mean diameter of spheroids.Exogenous apelin-13 treatment increased significantly and dose-dependently the migration ability of the cells compared with control untreated cells. The F13A in itself enhanced the migration ability of the cells to a small extent. F13A and apelin-13 in 1:1 proportion decreased the considerable stimulating effect of apelin-13 significantly.In endothelial tube formation assay treatment with apelin-13 and F13A increased significantly the mean length of tubes compared with control untreated cells.Inapoptosisassay, theexogenousapelintreatmentdecreasedsignificantlythenumber of apoptoticnucleiwhichwereinducedwith UV light. Western blot analysis showed that apelin-13 enhanced the levels of phosphorylated Erk1/2 and Akt molecules. The peak activation of both proteins occurred at 15 minutes.

Characterization of the spheroid formation capacity of the mesothelioma cell lines, and the effect of mTOR inhibition on the spheroid formation.

Next, we studied the spheroid formation capacity of 15 mesothelioma cell lines. We found that 6 cell lines of 15 showeddefinite, sphere-like construction. The effect of mTOR inhibition by temsirolimus was investigated on the 3-dimensional cell culture of 2 cell lines (SPC 111 and SPC 212). Temsirolimus treatment already after four days caused progressive loss of MPM spheroid integrity and significantly decreased number as well as mean dimeter of spheroids.

CONCLUSIONS

1. The NSCLC cell lines expressed the apelin and its receptor, although exogenous apelin treatment or transfection with apelin encoding vector did not stimulate the in vitro proliferation of NSCLC cells.
2. Increasing apelin levels by gene transfer to NSCLC cells significantly stimulated tumor growth compared with control tumors. Microvessel density and tumor capillary perimeters were significantly higher in the apelin-overexpressing tumors compared with controls.
3. Apelin expression levels were significantly increased in human NSCLC samples compared with normal lung tissue. High apelin protein levels were associated with elevated microvessel densities and poor overall survivor.
4. Our results reveal apelin as a novel angiogenic factor in human NSCLC. Moreover, it also provides the first evidence for a direct association of apelin expression with clinical outcome in a human cancer.
5. The APJ receptor was expressed in human lymphatic endothelial cells. Exogenous apelin treatment did not influence the in vitro proliferation of thecells, but it increased the migration, the mean length of tubes in tube formation assay, the relative number of spheroids in 3-dimensional culture of the cells, and decreased significantly thenumber of apoptoticnucleiwhichwereinducedwith UV light. Exogenous apelin treatment stimulated the Erk and PI3-K/Akt signaling pathways in the human lymphatic endothelial cells. Based on our in vitro experiments with the human lymphatic endothelial cells, apelin can also exert lymphangiogenic effects.
6. The mTOR inhibition by temsirolimus decreased significantly the number as well as the mean diameter of spheroids in the 3-dimensional cultures of the MPM cell lines. So it is apparent that the temsirolimus has a distinct impact on the tumor-initiating cell compartment of MPM cell lines. We first reported that

mTORinhibition distinctly attenuates human MPM growth in vivo as xenografts in SCID mice. These data implicate the mTOR inhibition might be a promising strategy for treating human MPM.

PUBLICATIONS

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1. Kenessey I, Keszthelyi M, Krámer Z, **Berta J**, Adám A, Dobos J, Mildner M, Flachner B, Cseh S, Barna G, Szokol B, Orfi L, Kéri G, Döme B, Klepetko W, Tímár J, Tóvári J. Inhibition of c-Met with the specific small molecule tyrosine kinase inhibitor SU11274 decreases growth and metastasis formation of experimental human melanoma. *Curr Cancer Drug Targets.*2010 May;10(3):332-42. IF: 4.771
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