Abstract  Background and aims: Recently we demonstrated that phosphatidylinositol 3-kinase (PI3K) is overexpressed in human lung cancer. This study evaluated whether the PI3K inhibiting agent wortmannin affects proliferation of human lung cancer cells in vitro and in vivo.  Methods: Effects of exposure of human non-small-cell lung cancer (NSCLC) cells (KNS-62, Colo-699) to wortmannin were investigated in vitro by proliferation, cytotoxicity, and DNA fragmentation assays. In vivo we examined the effects of blocking PI3K by wortmannin prior to xenotransplantation of human NSCLC cells into SCID-bg mice and the effect of systemic wortmannin administration following intrapulmonary xenotransplantation of human NSCLC.  Results: Exposure of KNS-62 and Colo-699 lung cancer cells to wortmannin inhibited proliferation in correlation to concentration in vitro. In vivo the blocking of PI3K by wortmannin prior to xenotransplantation caused a significant delay in the growth of subcutaneously induced tumors. Systemic wortmannin administration increased mean survival after intrapulmonary xenotransplantation of human NSCLC significantly by 38% and 47%.  Conclusions: These data suggest inhibition of PI3K activity as a potential target for treatment of human NSCLC. Systemic toxicity of wortmannin requires development of improved PI3K inhibitors with favorable pharmacological properties.  Keywords  Human lung cancer · Intrapulmonary xenotransplantation model · Phosphatidylinositol 3-kinase · Wortmannin

Introduction

We recently demonstrated that phosphatidylinositol 3-kinase (PI3K) is overexpressed in non-small-cell human lung cancer (NSCLC) [1]. PI3K is a heterodimer composed of a p85 regulatory subunit and a p110 catalytic subunit [2]. The products of PI3K function as second messenger molecules for regulation of cell growth, proliferation, and apoptosis in response to various growth hormones [3]. PI3K is required for oncogenic tyrosine kinases/receptors to induce tumor cell growth and proliferation [4, 5, 6, 7, 8, 9, 10, 11, 12, 13]. The p85 and p110 subunits of PI3K are overexpressed at protein level in primary lung carcinomas irrespective of the histological type, and PI3K overexpression is correlated with tumor grading [1]. In contrast, no overexpression is observed in normal lung tissue or benign lesions. PI3K has been identified as a potential target for anticancer drug development because of its role as a component of growth factor and oncogene activated signaling pathways [14]. Wortmannin (C23H24O8), a highly cell permeable fungal metabolite from Penicillium fumiculosum, acts as a selective, irreversible inhibitor of the catalytic p110 PI3K subunit [15, 16, 17, 18].

As PI3K is overexpressed in NSCLC, the aim of this study was to evaluate whether selective inhibition of the PI3K activity by its inhibitor wortmannin affects proliferation of human lung cancer cells in vitro and in vivo.
**Material and methods**

**Cell lines and culture conditions**

Human lung cancer cells KNS-62 [19] derived from the brain metastasis of a squamous cell carcinoma of the lung and Colo-699 [20] derived from adenocarcinoma of the lung were cultured in RPMI 1640 supplemented with 10% fetal calf serum, 1% sodium pyruvate, and 1% L-glutamine (Life Technologies, Paisley, UK). Cells grew as monolayers in 75 cm² flasks at 37°C in a humidified incubator gassed with 5% CO₂ and 95% air. When monolayers grew to approximately 80% confluence, cells were subcultured or harvested using trypsin EDTA (Life Technologies). Cells to be xenotransplanted were equilibrated in serum-free culture medium at a density of approximately 1×10⁶ cells per 50 µl injection volume. Cell viability was tested by trypan blue staining. All cell cultures were confirmed to be free of *Mycoplasma* infection by reverse transcriptase polymerase chain reaction of supernatants from densely growing cells following the instructions of the manufacturer (Takara Shuzo, Japan).

**[³H]Thymidine incorporation assay**

Tumor cells (1×10⁴; KNS-62, Colo-699) were seeded in 100 µl culture medium in 96-well microtiter plates. Cells were exposed to wortmannin (Sigma-Aldrich, Munich, Germany) in concentrations ranging from 10 to 400 nM for 24 or 48 h. To each well we added 0.02 µCi tritiated thymidine (43.0 Ci/mmol, Amersham Pharmacia Biotech, Braunschweig, Germany) 3 h before cell harvest. After washing with phosphate-buffered solution (PBS) three times cells were lysed by incubation with trypsin EDTA. The lystate was filtrated through a glass fiber filtermat (Wallac Oy, Turku, Finland) by a 96-well cell harvester (Inotech). The filtermat was placed in a MicroBeta sample bag together with 4.5 ml scintillation fluid. Counting was performed in a 1450 MicroBeta scintillation counter (Wallac Oy).

The relative recovery of incorporated [³H]thymidine in the DNA of proliferating cells was estimated for determination of inhibition of proliferation. Each measurement was performed six times.

**Cell viability assay**

To determine wortmannin-induced cytotoxicity cell viability assays were performed following the manufacturer’s instructions (EZ4U, Biomedica, Vienna, Austria). Tumor cells (5×10⁴; KNS-62, Colo-699) in 200 µl culture medium per well were seeded in 96-well microtiter plates. Cells were exposed to CA-4PD in concentrations ranging from 10⁻⁸ to 10⁻⁴ M for 3 or 24 h. Cell viability was estimated photometrically. Each measurement was performed six times.

**DNA fragmentation assay (JAM assay)**

The DNA fragmentation test was performed as originally described [21]. In 96-well microtiter plates we seeded 1×10⁴ cells in 100 µl culture medium per well. Cells were incubated for 3 h with 0.05 µCi tritiated thymidine. Medium was removed, and cells were washed twice in PBS. Cells were then incubated with wortmannin in concentrations ranging from 10 to 400 nM for 24 or 48 h. Adherent and floating cells were lysed by 100 µl 0.2% sodium dodecyl sulfate buffer. The lystate was processed as described above. The proportion of initially incorporated label was quantified. As fragmented DNA of apoptotic cells can pass the filtermat, the proportion of apoptotic cells can be estimated by the loss of initially incorporated label. Each sample was prepared six times.

**Experimental animals**

Pathogen-free female SCID-bg mice (Harlan Winkelmann, Borchen, Germany) [22] were maintained in sterile polycarbonate microisolator cages under pathogen-free conditions, fed autoclaved food and water ad libitum, and handled under stringent sterile conditions in a laminar flow hood.

To test whether inhibition of PI3K activity affects tumor cell proliferation in vivo tumor cells were incubated with wortmannin for 6 h prior to xenotransplantation at a concentration of 100 nM. Tumor cells were isolated, washed in PBS, and adjusted to 2×10⁶ tumor cells per 50 µl serum-free culture medium. For the induction of subcutaneous tumor growth 2×10⁶ tumor cells (KNS-62, Colo-699) were injected dorsally into the subcutis of the left flank. Animals were randomly distributed into groups of seven animals each and received tumor cells either pretreated by wortmannin or by PBS alone, which served as control.

Animals were monitored daily for local tumor growth, and tumors were gauged percutaneously in two diameters. Tumor volume was calculated by: \( V = \pi \times \frac{W_1 \times W_2}{2} \), where \( V \) is volume, \( L \) is length, \( W_1 \) and \( W_2 \) are width [23]. Animals were killed 28 days after subcutaneous xenotransplantation by CO₂ inhalation and tumors removed for histological examination.

An orthotopic xenotransplantation model was used to determine the systemic effect of wortmannin administration on the growth of human lung cancer in vivo. For induction of intrapulmonary tumor growth mice were anesthetized by intraperitoneal injection of 240 mg/kg Avertin [24], and 2×10⁶ tumor cells were introduced below the visceral pleura as described previously [20].

Animals were randomly distributed into treatment groups and control groups of seven animals each. Treatment was started on day 2 postoperatively by daily systemic administration of 1 mg/kg wortmannin intraperitoneally. Animals serving as controls were injected by sterile PBS in corresponding volumes. All animals were monitored daily for signs of respiratory distress or physical discomfort. When animals became respiratory insufficient, they were killed by CO₂ inhalation, and tumors were removed for histological examination.

All animal experiments were approved by the Review Board of the Ministry of Schleswig-Holstein, and the guidelines of Interdisciplinary Principles and Guidelines for the Use of Animals in Research, Testing and Education issued by the New York Academy of Sciences were observed strictly.

**Histological examination**

Tissues were fixed in formalin and stained with hematoxylin and eosin using standard procedures. For immunohistochemistry fresh tumor tissue was snap frozen in liquid nitrogen. Antibodies to PI3K p85 (Z-8) and p110 (C-17) were obtained from Santa Cruz Biotechnology (Heidelberg, FRG). Staining and immunohistochemical scoring was performed as published previously [1].

**Immunocompetence assay**

When animals were killed, blood was withdrawn from the abdominal vein, allowed to clot and serum was isolated by centrifuge. Cryopreserved histological sections of the corresponding tumor were fixated by acetone, air dried and incubated at 4°C overnight with 50 µl murine serum. Sections were washed in PBS for three times and incubated for 1 h with a peroxidase coupled rabbit anti-mouse IgG and IgM antibody in 1:1000 dilution (Dianova, Hamburg, Germany). After careful rinsing by PBS staining was performed adding peroxidase H₂O₂ diaminobenzidine substrate solution for 10 min. None of the animals had gained significant immunocompetence during the in vivo studies.