PhD Thesis

Mechanisms of hypoxia-induced chemoresistance – role of IAP family members and VEGF

submitted by

Dr. med. univ. Christoph Wohlkönig

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Department of Internal Medicine
Division of Pulmonology

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Univ. Prof. Dr. Horst Olschewski

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Declaration

I hereby declare that this thesis is my own original work and that I have fully acknowledged by name all of those individuals and organisations that have contributed to the research for this thesis. Due acknowledgement has been made in the text to all other material used. Throughout this thesis and in all related publications I followed the guidelines of “Good Scientific Practice”.

Please note that parts of this thesis have been published in “Hypoxia-induced cisplatin resistance is reversible and growth rate independent in lung cancer cells.“ Cancer Lett. 2011;308:134-43.

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My best friends who never make me feel bad about how less time I have left for them.

This thesis is also dedicated to my mother and my father who died two years ago...much too early. Without you there would be no me.

Summary

Lung cancer is the leading cause of cancer death worldwide. Non-small cell lung cancer (NSCLC) accounts for about 80% of all lung cancers. Platinum-based chemotherapy represents the gold standard for treating NSCLC. However, the efficacy is limited partially due to primary chemotherapy resistance. Resistance to cisplatin is highly complex and may occur due to various reasons i.e. decreased accumulation of cisplatin or increased repair of DNA damage. In addition, hypoxia-induced chemotherapy-resistance may play a role. It is well acknowledged that in solid tumors hypoxic conditions prevail and that this contributes to treatment failure and bad prognosis. Hypoxia was found to induce apoptosis and thereby select for multi-resistent cells but also hypoxia-induced adaptive mechanisms leading to chemotherapy resistance have been described. However, the exact downstream mechanisms of hypoxia-induced chemotherapy resistance remain to be elucidated.

For this PhD thesis we created an NSCLC cell line model which suggested that the growth rate of NSCLC cells is diminished in hypoxia but cells do not undergo apoptosis in hypoxia *per se*. However, NSCLC cells in hypoxia develop a strong resistance to cisplatin-induced apoptosis. We showed for the first time that re-oxygenation in NSCLC cell lines reversed cisplatin resistance after about 24 h.

This indicates a reversible hypoxia-specific cellular adaptation process leading to resistance against cisplatin. Hypoxia-induced growth rate reduction was mimicked by means of serum-starvation but did not induce cisplatin resistance. With this experiment we demonstrated that hypoxia-induced cisplatin resistance is independent of growth rate.

Furthermore we showed that hypoxia-induced chemotherapy resistance was associated with down-regulation of the pro-apoptotic BCL-2-family-protein BAX in A549 cells. Literature data suggested that the strongly hypoxia-regulated vascular endothelial growth-factor (VEGF) pathway might have pro-survival effects on cancer cells through an autocrine signalling loop and could thus play a role in hypoxia-induced chemoresistance. We showed that NSCLC cells expressed VEGF receptors and secreted VEGF, where the latter is indeed augmented in hypoxia. However, there was no pro-apoptotic role of autocrine VEGF signalling in NSCLC

lung cancer cell lines. This challenges the hypothesis from the literature that VEGF inhibitors act not only anti-angiogenic but also pro-apoptotic.

Zusammenfassung

Lungenkrebs ist noch immer die häufigste Todesursache weltweit. Der nicht-kleinzellige Lungenkrebs (NSCLC) macht ungefähr 80% aller Lungenkrebsarten aus. Die Platin-haltige Chemotherapie ist der Goldstandard in der Behandlung des NSCLC. Der Therapieerfolg ist aber durch einige Faktoren limitiert. Darunter auch die oft auftretende Resistenz der Tumorzellen gegen Chemotherapeutika. Die Mechanismen der Resistenz gegen das weltweit am häufigsten verwendete Chemotherapeutikum Cisplatin sind hoch komplex und vielfältig, darunter z.B. zu geringe Akkumulierung des Wirkstoffes in den Zellen oder verstärkte Mechanismen der DNA-Reparatur. Außerdem spielt die hypoxie-induzierte Chemotherapieresistenz eine Rolle. Es ist inzwischen gut belegt, dass in soliden Tumoren meist sauerstoffarme Bedingungen vorherrschen und dass dieser Umstand zum Therapieversagen und schlechterer Prognose für den Patienten beiträgt. Es wurde dabei einerseits gefunden, dass Hypoxie Krebszellen in die Apoptose treiben kann und damit nur apoptose- und damit auch meist multi-resistente Zellen überleben lässt. Andererseits gibt es auch Beweise dafür, dass sich Krebszellen auch an die hypoxischen Verhältnisse anpassen können und dabei auch Resistenzmechanismen entwickeln. Die genauen Mechanismen der hypoxie-induzierten Chemotherapieresistenz sind jedoch noch nicht ausreichend erforscht.

Im Rahmen dieser Dissertation haben wir ein NSCLC- Zellkulturmodell entwickelt das darauf hindeutete, dass die Wachstumsrate von NSCLC Zellen in der Hypoxie zwar deutlich eingeschränkt ist, die Zellen aber weder in die Apoptose noch in die Nekrose getrieben werden. Dennoch entwickelten die Zellen eine starke Resistenz gegen Cisplatin-induzierte Apoptose. Mittels Reoxygenierungsexperimenten ist es uns gelungen zum ersten Mal für NSCLC Zellen *in vitro* zu zeigen, dass diese Resistenz ein reversibler Prozess ist.

Diese Ergebnisse zeigen eindeutig, dass ein reversibler hypoxie-spezifischer adaptiver Prozess auf zellulärer Ebene zur Cisplainresistenz in NSCLC Zellen führt. Durch Entzug von Nährstoffen (Serum) bei normoxischen Zellen, konnte

deren Wachstumsrate auf das Niveau von hypoxischen Zellen gebremst werden. Trotz dieser niedrigen Teilungsrate blieb die Empfindlichkeit gegen Cisplatin erhalten. Dies zeigt, dass hypoxie-induzierte Cisplatinresistenz nicht durch die niedrige Wachstumsrate in der Hypoxie zu erklären ist. Weiters konnten wir im Rahmen dieses Projektes zeigen, dass die hypoxie-induzierte Chemotherapieresistenz in A549 Zellen mit einer Hemmung der Expression des pro-apoptotischen Proteins BAX, welches zur Bcl-2 Familie gehört, assoziiert war.
In letzter Zeit akkumulierte außerdem Evidenz, dass der stark hypoxie-induzierte Signalweg des vaskulären endothelialen Wachstumsfaktors (VEGF) durch einen autokrinen Regelkreis apoptosehemmende Effekte auf Krebszellen haben und somit eine Rolle in der hypoxie-induzierten Chemotherapieresistenz spielen könnte. In dieser Arbeit konnten wir zeigen, dass NSCLC Zellen sowohl Rezeptoren für diesen Wachstumsfaktor exprimieren, als auch selbst VEGF sezernieren und letzteres durch Hypoxie verstärkt wird. Ein Überlebensvorteil für Krebszellen durch einen möglichen autokrinen Regelkreis von VEGF konnte dagegen nicht festgestellt werden. Diese Daten stehen somit in Konflikt mit den in der Literatur beschriebenen pro-apoptotischen Effekten durch Unterbrechung des autokrinen VEGF-Regelkreises in Tumorzellen.

Table of Contents

Abbreviations

5-FU	Fluorouracil (5-fluoro-1H-pyrimidine-2,4-dione)
A1/BFL1	BCL2-related protein A1
ACTB	Beta-actin
AKT/PKB	Protein kinase B
ANOVA	Analysis of variance
Apaf-1	Apoptotic protease activating factor - 1
ARNT	Aryl hydrocarbon receptor nuclear translocator
ATP	Adenosintriphosphate
BAD	BCL-2-associated death promoter
BAK	BCL-2 homologous antagonist killer
BAX	BCL-2–associated X protein
BCL-2	B-cell lymphoma 2 protein
BCL-W/BCL2L2	BCL-2-like protein 2
BCL-XL	B-cell lymphoma-extra large
BH3	BCL-2 homology domain 3
BID	BH3 interacting-domain death agonist
BIK/NBK/BLK	BCL-2-interacting killer
BIM	BCL-2-like protein 11
BME	β-mercaptoethanol
BMF	BCL-2-modifying factor
BOK/MTD	BCL-2-related ovarian killer protein
BSA	Bovine serum albumine
cDNA	complementary DNA
CED-9	C. elegans death protein 9
CREB	cAMP response element-binding
DAG	Diacylglycerol

DAPI	4',6-diamidino-2-phenylindole
DD	Death domain
DED	Death effector domain
DIABLO	Direct IAP Binding Protein with Low PI
DMEM	Dulbecco’s modified Eagle's medium
DNA	Deoxyribonucleic acid
EGL-1	EGg Laying defective (C. elegans)
ELISA	Enzyme-linked immunosorbent assay
EPO	Erythropoietin
ERK ½	Extracellular-signal-regulated kinases
ET-1	Endothelin 1
FCS	Fetal calf serum
GRB2	Growth factor receptor-bound protein 2
HIF-1	Hypoxia-inducible factor 1
HOX	Hypoxia
HPRT1	hypoxanthine-guanine phosphorybosyltransferase
HRK/DP5	Activator of apoptosis harakiri
HRP	Horseradish peroxidase
HSP-90	Heat shock protein 90
IAP	Inhibitor of apoptosis protein
IgG	Immunoglobulin G
LDH-A	Lactate dehydrogenase A
MCL-1	Induced myeloid leukemia cell differentiation protein
MEK ½	Mitogen-activated protein kinase kinase
mRNA	messenger Ribonucleic acid
NOS	Nitric oxide synthase
NOX	Normoxia (ambient oxygen)
NOXA	Latin for “an injury”
NSCLC	Non-small cell lung cancer

PBS	Phosphate buffered saline solution
PHD	Prolyl-hydroxylase-domain carrying proteins
PI3K	Phosphoinositide 3-kinase
PIP2(3)	Phosphatidylinositol (3,) 4,5-bi(tri)sphosphate
PKC	Protein kinase C
PLC	Phospholipase C
PUMA/BBC3	p53 upregulated modulator of apoptosis/ BCL-2-binding component 3
qRT-PCR	Quantitative real-time polymerase chain reaction
Raf1	Proto-oncogene serine/threonine-protein kinase
RPMI	Roswell Park Memorial Institute medium
SCLC	Small-cell lung cancer
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
SHC	Src homology 2 domain containing transforming protein 1
siRNA	Small interfering RNA
SMAC	Second Mitochondria-derived Activator of Caspases
SOS	guanine nucleotide exchange factor “Son Of Sevenless”
SRC	Acronym of “sarcoma”
STARV	Starvation
TBS-T	Tris-buffered saline solution+Tween-20
TNF	Tumor necrosis factor
TRAIL	TNF-related apoptosis-inducing ligand
TUNEL	Terminal deoxynucleotidyl transferase dUTP nick end labelling
VEGF	Vascular endothelial growth factor
VEGFR	Vascular endothelial growth factor receptor
VHL	von-Hippel-Lindau protein
WHO	World Health Organisation

1. Introduction

1.1. Epidemiology of lung cancer

In about 36.000 people per year in Austria cancer is diagnosed. Statistically, in men it will be most likely prostate cancer followed by lung cancer and colon cancer. Before 1994, lung cancer was the most common cancer-entity in Austria. In women the lonely leader is breast cancer followed by uterus and colon cancer. Lung cancer represents only the fourth place regarding incidence. Nevertheless, concerning mortality, lung cancer catches up to the second place in females and is by far the most common cause of cancer death in males. It is good news that compared to the year 1997 the age-standardized incidence-rate of cancer overall has declined by 15% in men and women.

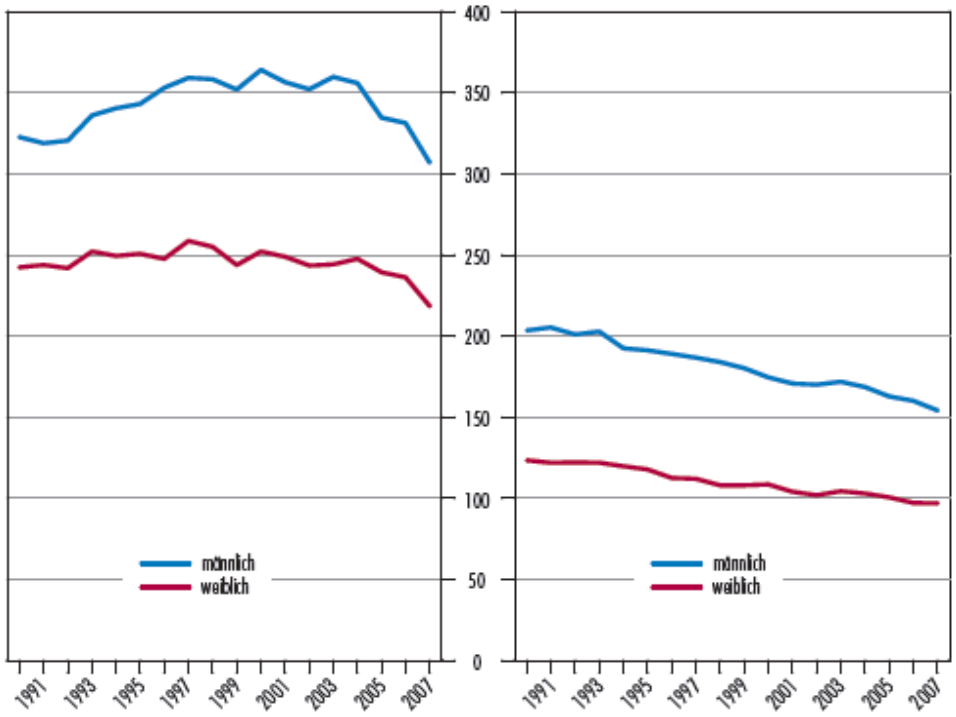


Fig. 1. Incidence (left) and mortality (right) of malignoma in Austria since 1990. Age-standardized rate per 100.000 people shown. Blue line represents men, red line represents women. Source: Statistik Austria, „Österreichischen Krebsregister (27.08.2009) und Todesursachenstatistik“.

However, this is not true for single entities. In females, lung cancer incidence and mortality were rising (+23% and +20% respectively) in the last decade, whereas in

men they were declining (-20% and -22%) (Statistik Austria). If this trend continues, the lung cancer mortality in women will surpass the rate in men within the next 15 years.

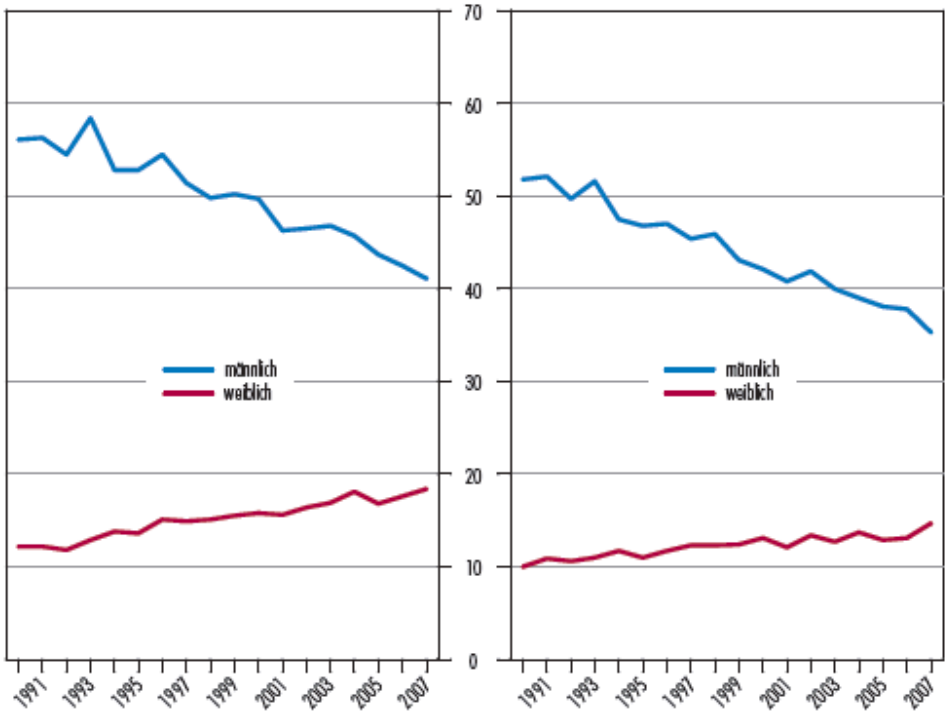


Fig. 2. Incidence (left) and mortality (right) of lung cancer in Austria since 1990. Age-standardized rate per 100.000 people shown. Blue line represents men, red line represents women. Source: Statistik Austria, „Österreichischen Krebsregister (27.08.2009) und Todesursachenstatistik".

The reason for this development, might be related to the changing smoking habits in Austrian women since the late 60-ies. While there were much less smoking young women than men in former days this ratio has almost equalled nowadays. The situation in Austria reflects the situation in the entire western world.

The correlation of tobacco smoking and lung cancer incidence has first been shown in the British Doctors Study, which was one of the first prospective clinical trials, running from the year 1951 to the year 2001, initiated by Richard Doll and Austin Bradford Hill. Innumerable publications followed indicating the carcinogenicity of tobacco smoking.

1.2. Classification of lung cancer

Clinically lung cancer is divided into two main entities, namely the small cell lung cancer (SCLC) and the non-small cell lung cancer (NSCLC). The reason for this differentiation is the clinically relevant difference between these two groups regarding tumor growth, invasiveness, metastasis spreading and treatment response. Accordingly, these entities receive a distinct treatment. The incidence of SCLC has been decreasing in the last decades. Currently, only about 13-17% of all lung cancers are SCLC. Interestingly it was also found that in diagnosed SCLC the proportion of women increased from 28% to 50% from 1973 to 2002 (Govindan, Page et al. 2006). This might be due to the changing smoking behaviour but it is also possible that changes in the pathologic classification of SCLC contributes to this (Ettinger, Aisner 2006).

Around 85% of all lung cancers are classified as NSCLC, which is a subsumption of a heterogeneous group of malignoma in the lung (Fig. 3). The most common entity in Austria and many other countries is adenocarcinoma. It is the entity that occurs most frequently in never smokers (and there mostly in women), although most cases are seen in smokers (Khuder 2001). Second are the squamos cell carcinomas. It was usually seen as the “central lung carcinoma” but in recent reports it was also found more frequently in the periphery of the lung (Funai, Yokose et al. 2003). The third group of NSCLC, the so called large cell carcinoma, comprises carcinomas which cannot be classified as SCLC, squamous- or adeno-carcinomas because they are too undifferentiated. These carcinomas account for about 9% of all lung cancers (Iyoda, Hiroshima et al. 2001). While large cell carcinomas and SCLC are equally distributed between genders, there is a significant difference for adeno- and squamous cell carcinomas. While adenocarcinoma comprises only about 28% of all lung cancers in men, it accounts for 42% in women. For squamous cell carcinomas it’s the other way round with 44% in men and 24% in women.

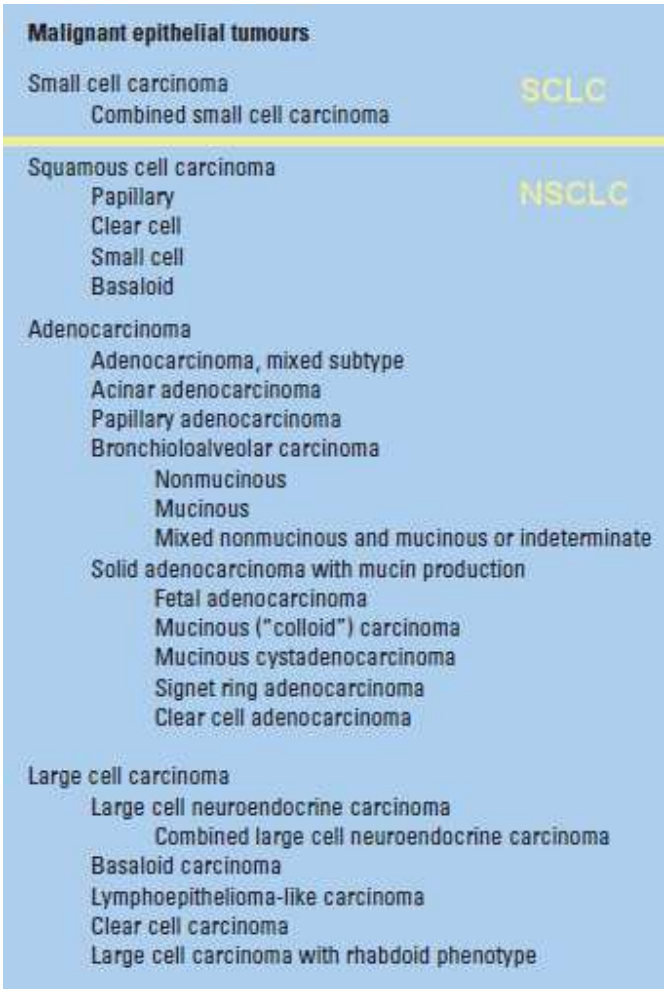
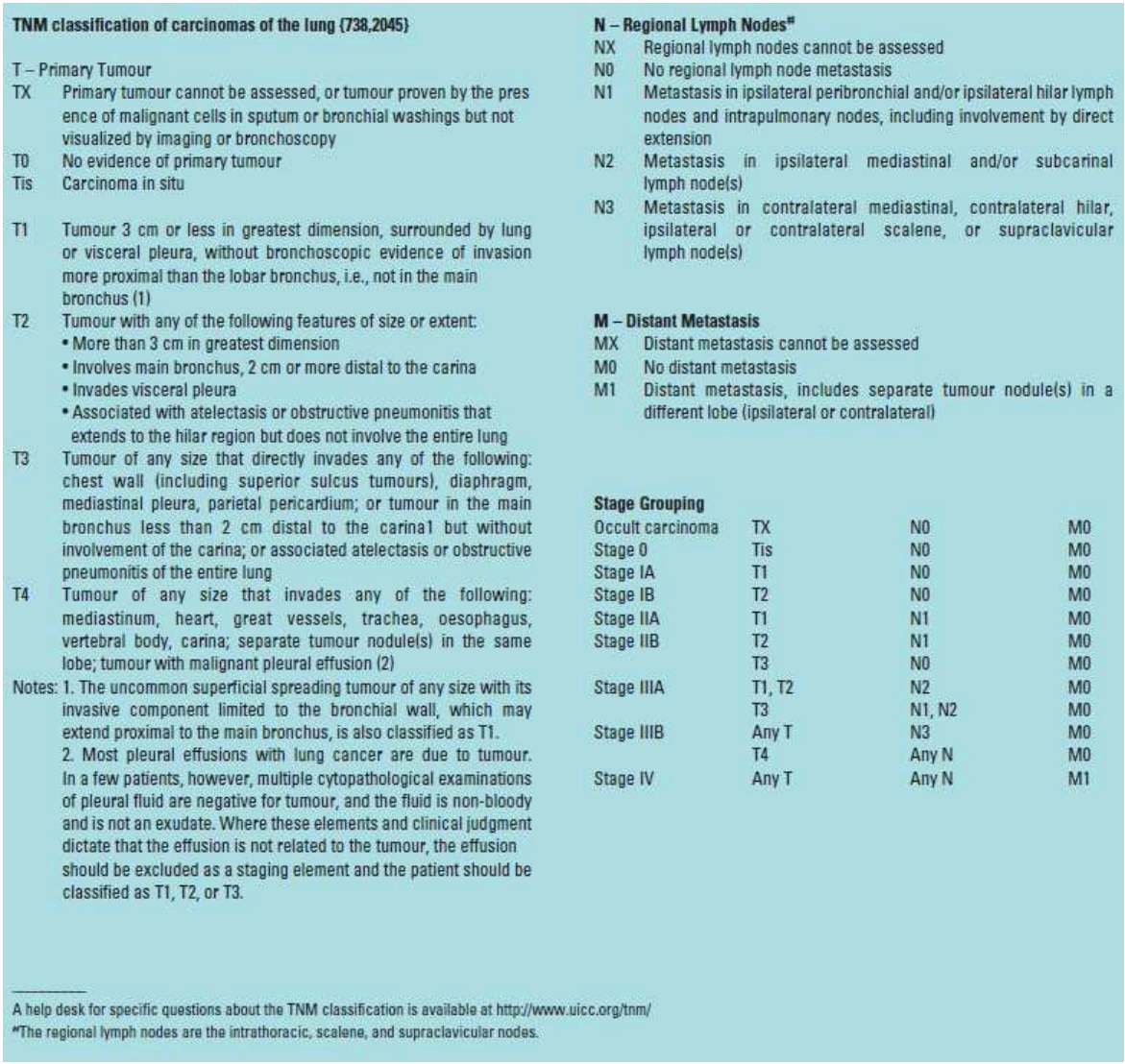


Fig. 3. Most common entities of lung cancer, adapted out of: Travis W.D., Brambilla E., Muller-Hermelink H.K., Harris C.C. (Eds.): World Health Organization Classification of Tumours. Pathology and Genetics of Tumours of the Lung, Pleura, Thymus and Heart. IARC Press: Lyon 2004

1.3. Treatment of lung cancer

To treat a patient with cancer to the best of the current knowledge the cancer has to be staged first. The staging system is a help for the physician to categorize the spread of the tumor and the operability. Knowing the stage the attending physician can choose the treatment regimen with the most promising outcome for the patient according to clinical studies. The system accepted and used in most developed countries is the TNM classification. It was developed by Pierre Denoix in the years

1943 to 1952. Since the year 1950 it is perpetuated by the Union Internationale Contre le Cancer, which published the 7th edition in 2009 (UICC). The classification is based on the size and localization of the primary tumor (T), the existence of lymph node metastasis (N) and distant metastasis (M). For details see Figure 4 (6th edition). The SCLC classification is usually simplified to “very limited” disease (half of the thorax), “limited” disease (thorax) and “extensive” disease with extrathoracical metastasis.



TNM classification of carcinomas of the lung {738,2045}

T – Primary Tumour

TX Primary tumour cannot be assessed, or tumour proven by the pres ence of malignant cells in sputum or bronchial washings but not visualized by imaging or bronchoscopy

T0 No evidence of primary tumour

Tis Carcinoma in situ

T1 Tumour 3 cm or less in greatest dimension, surrounded by lung or visceral pleura, without bronchoscopic evidence of invasion more proximal than the lobar bronchus, i.e., not in the main bronchus (1)

T2 Tumour with any of the following features of size or extent:
- More than 3 cm in greatest dimension
- Involves main bronchus, 2 cm or more distal to the carina
- Invades visceral pleura
- Associated with atelectasis or obstructive pneumonitis that extends to the hilar region but does not involve the entire lung

T3 Tumour of any size that directly invades any of the following: chest wall (including superior sulcus tumours), diaphragm, mediastinal pleura, parietal pericardium; or tumour in the main bronchus less than 2 cm distal to the carina1 but without involvement of the carina; or associated atelectasis or obstructive pneumonitis of the entire lung

T4 Tumour of any size that invades any of the following: mediastinum, heart, great vessels, trachea, oesophagus, vertebral body, carina; separate tumour nodule(s) in the same lobe; tumour with malignant pleural effusion (2)

Notes: 1. The uncommon superficial spreading tumour of any size with its invasive component limited to the bronchial wall, which may extend proximal to the main bronchus, is also classified as T1.
2. Most pleural effusions with lung cancer are due to tumour. In a few patients, however, multiple cytopathological examinations of pleural fluid are negative for tumour, and the fluid is non-bloody and is not an exudate. Where these elements and clinical judgment dictate that the effusion is not related to the tumour, the effusion should be excluded as a staging element and the patient should be classified as T1, T2, or T3.

N – Regional Lymph Nodes#

NX Regional lymph nodes cannot be assessed

N0 No regional lymph node metastasis

N1 Metastasis in ipsilateral peribronchial and/or ipsilateral hilar lymph nodes and intrapulmonary nodes, including involvement by direct extension

N2 Metastasis in ipsilateral mediastinal and/or subcarinal lymph node(s)

N3 Metastasis in contralateral mediastinal, contralateral hilar, ipsilateral or contralateral scalene, or supraclavicular lymph node(s)

M – Distant Metastasis

MX Distant metastasis cannot be assessed

M0 No distant metastasis

M1 Distant metastasis, includes separate tumour nodule(s) in a different lobe (ipsilateral or contralateral)

Stage Grouping

Occult carcinoma	TX	N0	M0
Stage 0	Tis	N0	M0
Stage IA	T1	N0	M0
Stage IB	T2	N0	M0
Stage IIA	T1	N1	M0
Stage IIB	T2	N1	M0
	T3	N0	M0
Stage IIIA	T1, T2	N2	M0
	T3	N1, N2	M0
Stage IIIB	Any T	N3	M0
	T4	Any N	M0
Stage IV	Any T	Any N	M1

A help desk for specific questions about the TNM classification is available at http://www.uicc.org/tnm/

#The regional lymph nodes are the intrathoracic, scalene, and supraclavicular nodes.

Fig. 4. TNM classification of carcinomas of the lung out of: Travis W.D., Brambilla E., Muller-Hermelink H.K., Harris C.C. (Eds.): World Health Organization Classification of Tumours. Pathology and Genetics of Tumours of the Lung, Pleura, Thymus and Heart. IARC Press: Lyon 2004

Depending on the TNM “values” the disease is then classified into different stages (Fig. 4). According to the stage, a certain therapy regimen is recommended by guidelines. In Austria the “Interdisziplinäre S3-Leitlinie der Deutschen Gesellschaft für Pneumologie und Beatmungsmedizin und der Deutschen Krebsgesellschaft” (Goeckenjan, Sitter et al. 2011) is the most important guideline.

Up to stage IIIA (T3N1M0) the recommended treatment is surgical resection of the tumor (and regional lymph nodes). In some cases even patients with a stage IIIB (T4 N0/1) tumor might be eligible for operation. Nevertheless from stage IB on adjuvant (=after resection) chemotherapy (and/or radiotherapy) is recommended, while a neo-adjuvant (=before resection) chemotherapy was found to be of no benefit for the patients. When the disease reaches inoperable stages (IIIB-IV) chemotherapy remains the only treatment option, which was shown to have a significant benefit in survival and quality of life (Chemotherapy in non-small cell lung cancer: a meta-analysis using updated data on individual patients from 52 randomised clinical trials. Non-small Cell Lung Cancer Collaborative Group. 1995, Spiro, Rudd et al. 2004, Thongprasert, Sanguanmitra et al. 1999).

Independent of the stage of the tumor the gold standard chemotherapy, although it is in use since the early seventies, is cisplatin (cis-Diamindichloroplatin(II)) (Lippman, Helson et al. 1973, NSCLC Meta-analyses Collaborative Group, Arriagada et al. 2010). Cisplatin is used in different combinations with other chemotherapeutics. The best evidence for efficacy data has been gathered for the combination of cisplatin and vinorelbin (Goeckenjan, Sitter et al. 2011). The known modes of action of cisplatin are cross-linking DNA-strands, induction of point-mutations and inhibition of DNA-repair and telomerase activity. All this finally leads to the induction of apoptosis in cells with a high division rate.

1.4. Basics of apoptosis

Apoptosis is one type of programmed cell death, which is differentiated from the other types by the involvement of proteins called caspases. This family of proteases is now accepted as the initiators (Caspase 2, 8, 9 and 10) and effectors (Caspase 3, 6, and 7) of apoptosis. They were identified in humans by homologies to the apoptosis regulating proteins in C. elegans (for review see (Cohen 1997, Alnemri 1997). There are 12 caspases known but not all of them play a role in apoptosis. Through two main pathways apoptosis can be induced: The “intrinsic” and the “extrinsic” pathway.

The extrinsic pathway is invoked by ligands binding to receptors belonging to the group of so called “death receptors” e.g. TNF-receptor. Common to all these receptors is the “Death domain” (DD). Ligands for these receptors might be toxins, hormones, cytokines, etc. As the receptor is activated, adaptor-proteins are recruited where the last one harbours a “death effector domain” (DED). Pro-caspase 8 binds to DED and is catalytically changed to Caspase-8, which then initiates apoptosis by activating effector caspases (for review see (Adams 2003)).

Stress and damage of the cell activate the intrinsic pathway. This might be radiation, heat, hypoxia, nutrient deprivation, calcium influx due to membrane damage, DNA damage by chemotherapeutics and much more. This indicates that the intrinsic pathway is very complex and not everything is known. The involvement of the mitochondria seems to be common to all apoptosis mechanisms. Different signals induce a leakage of the outer mitochondrial wall leading to efflux of pro-apoptotic factors like cytochrome c or SMAC/DIABLO. Released cytochrome c binds to the apoptotic protease activating factor - 1 (Apaf-1). These two bind to pro-caspase 9, building a complex called “apoptosome”, which then activates pro-caspase by cleavage. SMAC/DIABLO binds and inhibits “inhibitor of apoptosis proteins” (IAPs), which suppress caspases.

It is obvious that this machinery needs a tight regulation. The cell is not obliged to death by the first signal. The process of apoptosis is divided into two phases. The first where the signal or stress is sensed, processed and the decision if the cells

has to die is made. This phase is reversible. If the pro-apoptotic signals outweigh the pro-survival signals, apoptosis will progress to the execution phase, which is no longer reversible. How this decision is made is still not entirely clear, but obviously one group of related proteins plays a major role: the BCL-2 family.

There are at least 20 members in this family which are subdivided into three groups: The BCL-2 subfamily is pro-survival and the BAX and BH3-only subfamilies act pro-apoptotic (see Fig. 5).

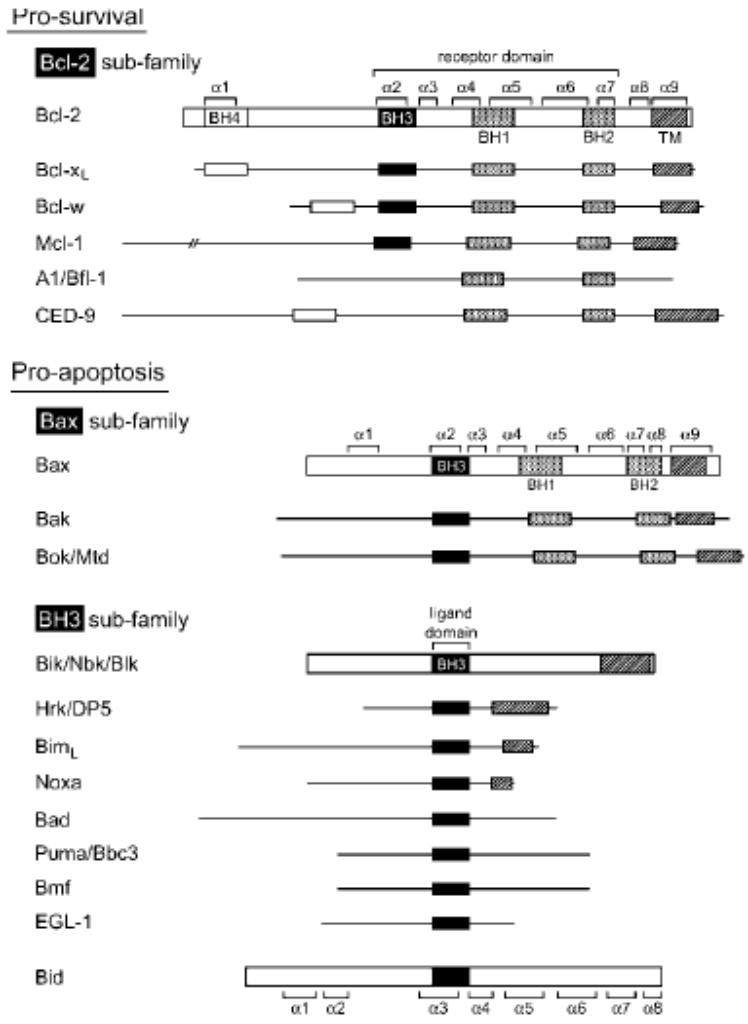


Fig. 5. Protein structure of the Bcl-2 sub-families. Out of Cory, Huang et al. 2003.

The BH-3 only proteins were found to be upstream of BCL-2 and BAX members and turn the scale for death or survival of a cell. They cannot induce apoptosis by themselves but inhibit BCL-2 members, the counterpart of BAX members. Thus the

decision if a cell goes into apoptosis is controlled by the balance between pro- (BAX) and anti-apoptotic (BCL-2) proteins (for review see (Cory, Huang et al. 2003))

1.5. Resistance to chemotherapy

As said before most chemotherapeutic drugs act against cancer by inducing apoptosis in the tumor cells. Unfortunately almost obligatory resistance to chemotherapy occurs. Either the tumor does not respond to therapy from the beginning (primary resistance) or develops resistance during chemotherapy (secondary resistance). Treatment resistance is highly complex. Searching only for the gold standard cisplatin brings up a fistful of causes for such resistance (for review see (Chu 1994, Borst, Rottenberg et al. 2008)).

It was thought for a long time that cisplatin enters the cells by passive diffusion (Perez 1998). Now there is growing evidence that Cu-transporters play a role in cisplatin resistance (Komatsu, Sumizawa et al. 2000, Song, Savaraj et al. 2004). However, how these transporters are involved is not clear at all. For some it's even believed that not the efflux of cisplatin by the transporter but a detoxification of cisplatin due to binding to the receptor is the mechanism (Dmitriev 2011). However, in resistant cells by trend there was less cisplatin found than in their wild-type counterparts (Hall, Okabe et al. 2008).

There is a correlation between glutathione levels and cisplatin resistance *in vitro*. Hence, it was proposed that the drug might be inactivated in a complex with glutathione. There were several ways proposed how glutathione may interact with cisplatin, however, the exact mechanism and the clinical importance are unclear (for review see (Chen, Kuo 2010). Also thioredoxin had a similar function in cisplatin resistance (Kelley, Basu et al. 1988).

As the main mechanism of action of cisplatin is thought to be forming DNA-adducts and point mutations, DNA-damage repair or tolerance should be the most important mechanism for cisplatin resistance. Indeed, this is confirmed in many papers (e.g. (Chu 1994, Masuda, Ozols et al. 1988)). It is a matter of debate if cisplatin-sensitive cells have lost DNA-repair or if resistant cells have an increased

repair. Nevertheless, DNA-damage repair is acknowledged as one of the most important mechanisms of cisplatin resistance but may very likely occur in combination with other mechanisms (Borst, Rottenberg et al. 2008).

Chemotherapeutic drugs are known to induce apoptosis. Obviously any mechanism that leads to an inhibition of apoptosis in tumor cells may lead to chemotherapy resistance as well. Unfortunately there is not the one way how treatment induces apoptosis and likewise there are numerous ways a cell can become resistant to apoptosis. However, there is one thing that drew a lot of attention in the last decade of tumor research and that is the microenvironment of cancer and there especially: hypoxia.

1.6. Hypoxia-induced chemotherapy resistance

It is well acknowledged that in solid tumors hypoxic conditions prevail and that this contributes to treatment failure and bad prognosis (Vaupel, Mayer 2007). The low oxygen tension is the inevitable result of the imbalance between rapid tumor growth and high metabolism and lagging vessel formation. There are two possibilities how hypoxia might induce resistance in cancer cells. On the one hand hypoxia causes cell death via apoptosis and thereby selects for apoptosis resistant cells [5–7]. On the other hand it was shown that adaptation to hypoxia leads to chemotherapy resistance [8–13]. The level of oxygen seems to be the critical factor deciding which mechanism comes to play [14]. At O2 concentrations below 0.1%, selection may be most important while at oxygen concentrations around 1% the literature is inconsistent [15,16]. Proliferation of cancer cells is inhibited by low oxygen tensions [17,18]. It is commonly believed that the potency of DNA-damaging agents, like cisplatin, to cause apoptosis is strongly dependent on the growth rate [19–21]. Thus, a reduction of growth rate by hypoxia could be, amongst others, an important mechanism leading to cisplatin resistance.

In the year 1991 Gregg Semenza et al. found hypoxia-inducible nuclear factors that bind to an enhancer element next to the human erythropoietin (EPO) gene and explained how EPO is upregulated under low oxygen tension (Semenza, Nejfelt et al. 1991). One year later he found that it´s merely one factor binding at the site for

transcriptional activation that is important for the regulation of EPO in hypoxia (Semenza, Wang 1992). Another year later Semenza and his colleague Wang created the term “hypoxia inducible factor 1 (HIF-1)” and proofed that this transcription factor is not unique to EPO but is part of a general mammalian cellular response to hypoxia (Wang, Semenza 1993). HIF-1 is a heterodimer consisting of the constitutively expressed HIF-1β, also known as aryl hydrocarbon receptor nuclear translocator (ARNT) and the subunit HIF-1α. Interestingly the alpha subunit is constitutively expressed and transcribed as well but under non-hypoxic conditions it is immediately ubiquitinated by the von-Hippel-Lindau protein (VHL). The sensing of hypoxia was shown to be done by prolyl-hydroxylase-domain carrying proteins (PHD), which then in turn deactivate VHL leading to a stable alpha subunit (Epstein, Gleadle et al. 2001).

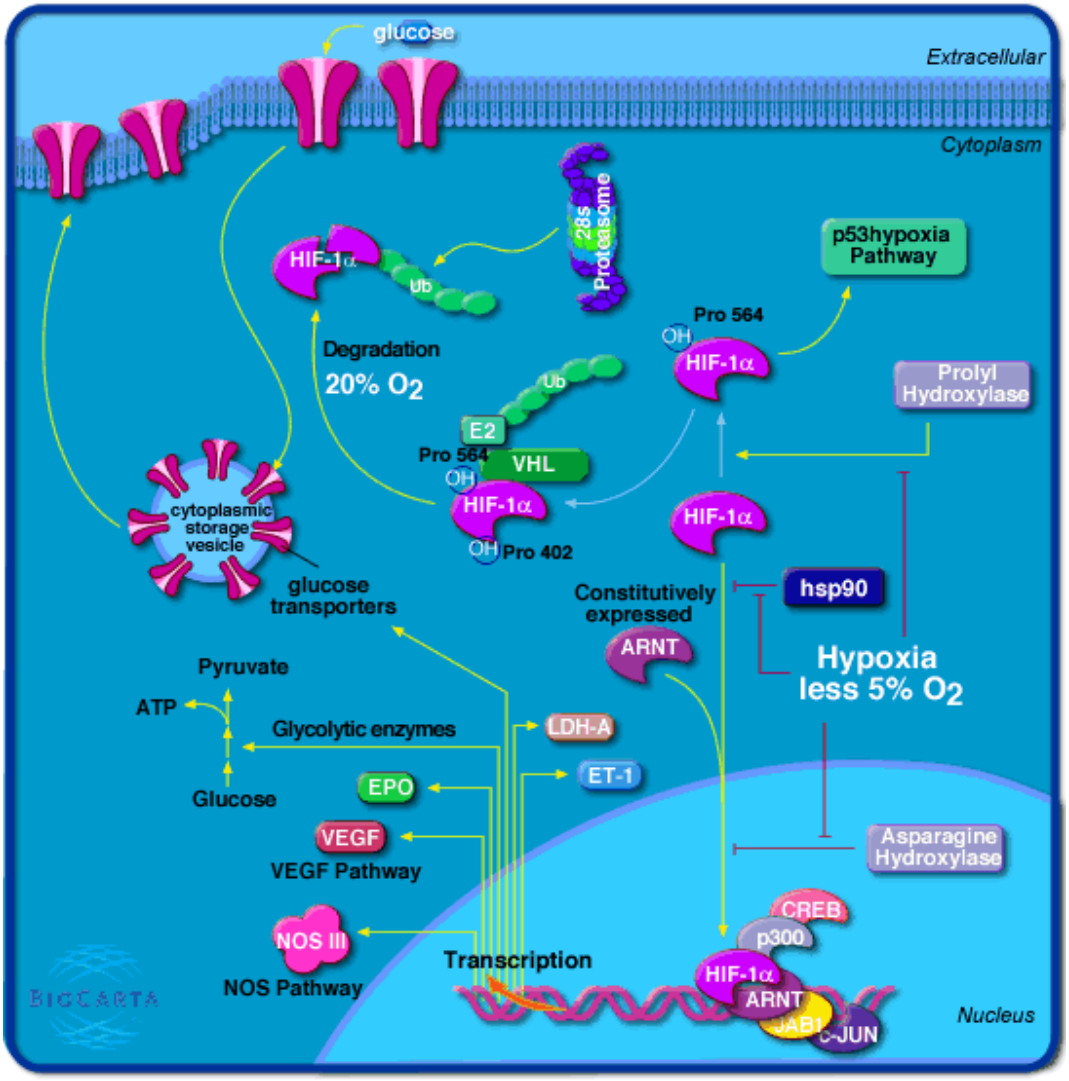


Fig. 6. Oxygen dependent regulation of HIF-1α. Source: http://www.biocarta.com/pathfiles/h_hifpathway.asp

Soon the role of HIF-1 in cancer and hypoxia-induced chemotherapy resistance was discovered (for review see (Semenza 2010a, Semenza 2010b, Giaccia 1996, Ke, Costa 2006). Hence it became of high interest as target for cancer therapy (Semenza 2010a, Semenza 2003, Greco, Marples et al. 2003). Clinical data showing correlation of overexpression of HIF-1 and poorer prognosis (Birner, Schindl et al. 2000) and *in-vitro* data (Brown, Cowen et al. 2006, Hussein, Estlin et al. 2006, Kilic, Kasperczyk et al. 2007, Wen, Ding et al. 2010, Zhang, Zhang et al. 2004) validated HIF-1 as a promising cancer target. However, it will take time until blockers for HIF-1 are available for clinicians provided that they really hold to be beneficial. Finally it has to be mentioned that also HIF independent pathways in hypoxia-induced chemoresistance have been published (Dong, Venkatachalam et al. 2001, Erler, Cawthorne et al. 2004, Piret, Cosse et al. 2006).

1.7. The role of vascular endothelial growth factor in lung cancer

In the year 1989 VEGF was first isolated from conditioned media of tumor cell lines and folliculostellate cells as a potent mitogen for endothelial cell and angiogenic factor (Ferrara, Henzel 1989, Plouet, Schilling et al. 1989). Out of the many downstream targets of HIF-1, VEGF was of interest for us, because a drug targeting VEGF was already approved for clinical use in lung and colon cancer. It is a humanized monoclonal antibody called bevacizumab (Avastin®). The mechanism of anti-cancer action proposed by the company is, however, not a direct effect on tumor cells but the inhibition of neo-angiogenesis that a tumor is evoking in the microenvironment by releasing this growth factor. The anti-angiogenic properties of bevacizumab were also reinforced by a number of mouse model studies (Ferrario, Gomer 2006, Fujita, Sano et al. 2007, Dai, Luo et al. 2007).

Nevertheless, further research revealed that VEGF can also act as autocrine growth factor for tumors expressing VEGF receptors (Masood, Cai et al. 2001, Castro-Rivera, Ran et al. 2004, Liu, Peng et al. 2009). Moreover, Epstein proposed in the year 2007 that VEGF signalling inhibitors might act even more pro-apoptotic

than anti-angiogenic (see also Fig. 7) (Epstein 2007). Furthermore, autocrine and intracrine signalling loops of VEGF promoting tumor growth, survival and even metastasis were suggested for melanoma and breast cancer cell models (Lee, Seng et al. 2007, Sini, Samarzija et al. 2008).

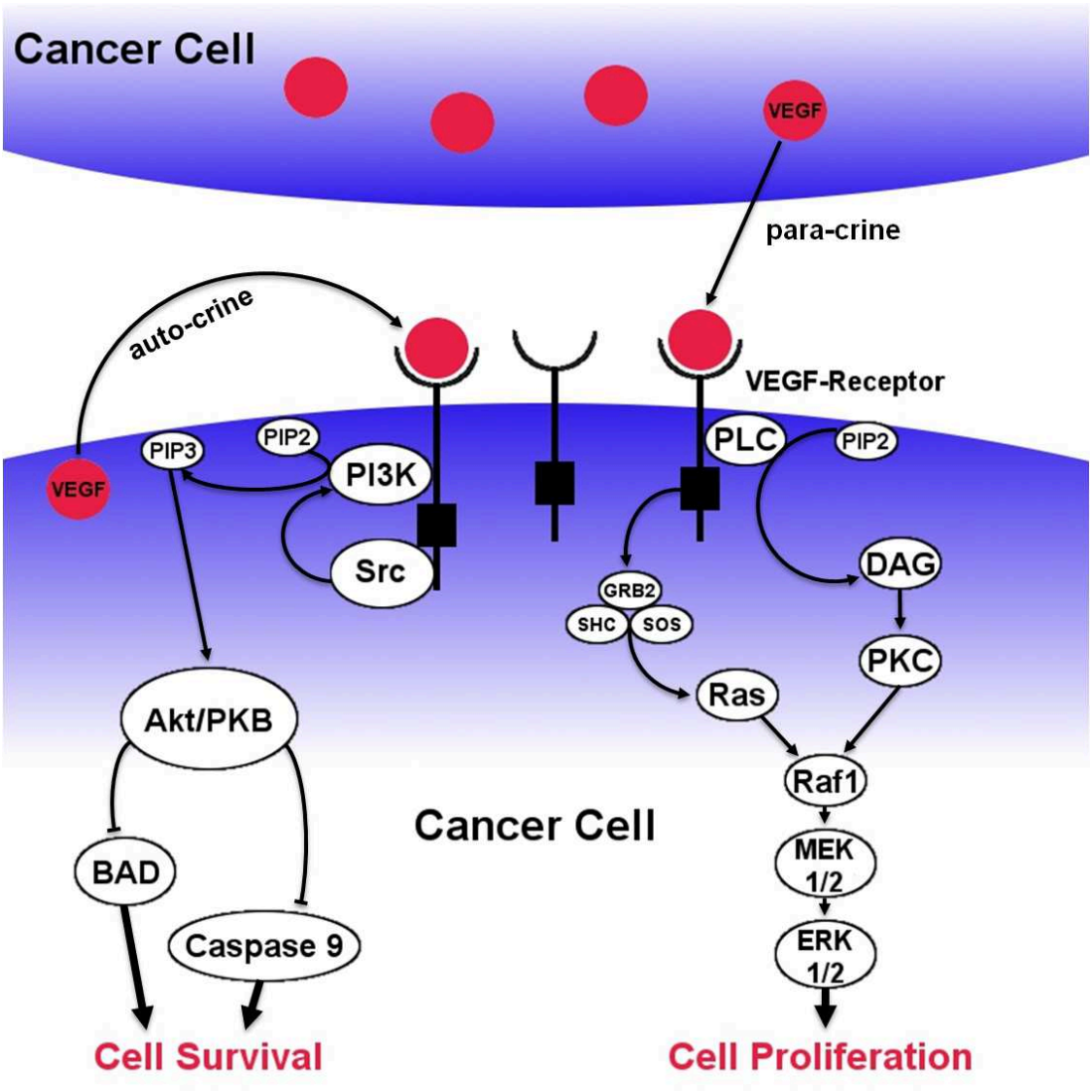


Fig. 7. Model of the putative auto- or para-crine loop of VEGF in cancer cells.

However, data confirming a putative effect of bevacizumab on cancer on the cellular level is insufficient because only available for colon and breast cancer models (Emlet, Brown et al. 2007, Calvani, Trisciuoglio et al. 2008). Data for the clinically approved target lung cancer has been lacking.

2. Aims and description of the study

First aim of this study was to investigate the time-frame, extent and reversibility of hypoxia-induced chemotherapy resistance in a NSCLC cell line model. Therefore, NSCLC cell lines were incubated at 1% oxygen, treated with cisplatin and analysed for cell survival, apoptosis and proliferation in different setups. The results were compared to control cells at ambient air oxygen concentration (21%). Re-oxygenation experiments should clarify the reversibility of this resistance.

Second aim was to explore the role of apoptosis-regulating proteins in our NSCLC cell line model of hypoxia-induced chemotherapy resistance. mRNA expression analysis and western blots of hypoxic versus normoxic cells were engaged to reach this aim.

Third aim was to examine the effects of the anti-VEGF antibody bevacizumab, used as anti-angiogenic drug in lung cancer, on the cellular level. Therefore, NSCLC cell lines were tested for the presence of an para- or auto-crine signalling loop of VEGF by means of western blots and immunofluorescence stainings. Effects on apoptosis or proliferation of NSCLC cells by interfering with this putative signalling loop were analyzed using thymidin-uptake assay and flow cytometry.

3. Materials and Methods

3.1. Chemicals and solutions

CHEMICALS	COMPANY
DMEM/F12	Gibco
RPMI 1640	ATCC
FCS	Biowest
L-glutamine	Gibco
Penicillin/Streptomycin	Gibco
N_2/CO_2	Air Liquide
[^{3}H]-thymidine	GE Healthcare
BSA	Jackson ImmunoResearch Laboratories
Trypsin	Gibco
Milkpowder	Bio-Rad
Tris	Sigma
Tween 20	Bio-Rad
Formalin	Donauchem
Triton X-100	Sigma
Cisplatin	Ebewe Pharma
Glycine	Sigma
SDS	Sigma
Acrylamide	Merck
Ammonium persulfate	Sigma
TEMED	Bio-Rad
B-mercaptoethanol	Sigma
Methanol	Merck
Ethanol	Merck

Ponceau S	Sigma

Self-made solutions:

Running buffer (10x):

Trizma Base 30 g

Glycine 144 g

SDS 10 g

to 1L with H_2O

Transfer buffer (10x):

Trizma Base 28 g

Glycine 143 g

to 1L with H_2O

Transfer buffer (1x):

H_2O 1575 ml

Methanol 450 ml

Transfer (10x) 225 ml

TBS (10x):

Trizma HCL 31.5 g

NaCl 80 g

to 1L H_2O, pH=7.5

Blocking buffer:

TBS 1x

Tween-20 0.1%

Milkpowder 5% (w/v)

SDS-Epho stripping:

Solution A

Tris 15.15 g

SDS 1 g

to 250 ml H_2O, pH=6.8

Solution B

SDS 20% in H_2O

Working

Solution A 12.5 ml

Solution B 5 ml

BME 350 µl

to 50 ml H_2O

3.2. Materials and devices

Cell culture	
Laminar flow	Hera Safe
Incubators	RS Biotech
Water bath	J.P. Selecta Unitronic OR
Hypoxic work station	BioSpherix Xvivo G300CL
Centrifuge	Heraeus Multifuge 1
Disposables	Corning®, BD Falcon®, Sarstedt®
Cell counting	CASY®
Proliferation	
Cell harvester	Perkin Elmer, Filtermate
Filterplates 96-well	Perkin Elmer, UniFilter GF/C
Beta-counter	Perkin Elmer, 1450 Microbeta Trilux Liquid

	Scintilation&Luminescence Counter
Apoptosis	
8-well chamber slides	Nunc
Microscope+camera	Olympus, BX51+DP71
Flow cytometer	BD FACSCalibur®
Western blot	
Blotting system	Bio-Rad Mini-Protean 3 Cell
Magnetic stirrer/Vortex	IKA, MSH basic, REO basic C, MS3 basic
Homogenizer	Hielscher UP50H
Centrifuge	Heraeus, Fresco 17
pH-meter	Hanna Instruments, pH 213
Developer	AGFA, Curix 60
Films	AGFA, T-MAT G/RA; GE Healthcare, Amersham Hyperfilm™ ECL
Membranes	Bio-Rad, Immun-Blot™ PVDF Membrane; Bio-Rad, Trans-Blot® Pure Nitrocellulose Membrane
RNA/PCR	
Cycler	Bio-Rad, MyCycler
Light-cycler	Applied Biosystems, ABI Prism 7000
Photometer	Eppendorf, BioPhotometer
ELISA	
Plate-washer	Tecan, microplate washer
Photometer	Molecular Devices, SpectraMax Plus

Commercial kits:

Roche, In Situ Cell Death Detection Kit, Fluorescein (TUNEL)
Merck, Caspase-3 Intracellular Activity Assay Kit I (PhiPhiLux® G1D2)
Roche, Complete mini
Sigma, RIPA buffer
Sigma, Laemmli buffer (2x)
Bio-Rad, Precision Plus Protein Standard, dual color
Qiagen, RNeasy mini kit
Qiagen, RNase-Free DNase Set
Fermentas, RevertAid™ H Minus First Strand cDNA Synthesis Kit
Immuno-Biological Laboratories, Human VEGF Assay Kit – IBL 96 Well

3.3. Cell culture

The human NSCLC cell lines NCI-H358, NCI-H23, NCI-H1299 and NCI-H441 were purchased from American Type Culture Collection (ATCC, Manassas, VA). The human NSCLC cell lines A549 and A427 were purchased from Cell Lines Service (CLS, Eppelheim, Germany). Cells were cultured in DMEM/F12 1:1 (Gibco, Paisley, UK) or RPMI 1640 (ATCC) culture medium supplemented with 10% fetal calf serum (FCS, Biowest, Nuaillé, France), 2 mM L-glutamine (Gibco), 100 U/ml penicillin, and 100 µg/ml streptomycin (Gibco) in a humidified incubator with 21% oxygen and 5% CO_2 at 37°C.

3.4. Hypoxic treatment

For performing experiments in hypoxia cells were cultured in the automated Xvivo system G300CL (BioSpherix, Lacona, NY) with an integrated humidified incubator at 37°C in an atmosphere containing 1% oxygen and 5% CO_2 with the help of N_2 and CO_2 (Air Liquide, Paris, France). Manipulation of cells (splitting, treatment with

drugs…etc.) in hypoxia was done in the integrated work chamber of the Xvivo system at 1% O_2 and 5% CO_2. In most experiments cells were preincubated for 3 days in hypoxia, this is referred to as “chronic hypoxic cells” in the manuscript.

3.5. Proliferation and viability

For electronic pulse area analysis, NSCLC cell lines ($2x10^5$ cells/dish) were plated in 35 mm petri dishes in duplicates and incubated in normoxia or hypoxia for up to 96 h. At indicated time points cells were collected by trypsinization including cells from supernatant and counted via electronic pulse area analysis (CASY®, Innovatis, Reutlingen, Germany). Along with the cell counts, viability of the cells was assessed. In addition, proliferation was determined with [^{3}H]-thymidine (GE Healthcare, Fairfield, USA) incorporation. NSCLC cells were plated in 96 well plates ($5x10^3$ cells/well) in normoxia, with and without serum starvation (1% FCS) or in medium supplemented with 1% FCS and 5 g/l bovine serum albumin (BSA, Jackson ImmunoResearch Laboratories, West Grove, USA) and hypoxia for 72 h. Other experiments were performed with reduced FCS as indicated. To some experiments human VEGF (Sigma-Aldrich, St. Louis, MO, USA), bevacizumab (Avastin®, Roche, Mannheim, Germany) or human normal immunoglobulin (Nanogam®, Sanquin, Asterdam, Netherlands) as control was added. Eighteen hours prior to the cell harvest, 2 µCi [^{3}H]-thymidine (GE Healthcare) was added per well. Cells were harvested with the Filtermate Harvester (Perkin Elmer, Waltham, USA) and proliferation was quantified with the 1450 Microbeta Trilux Liquid Scintilation&Luminescence Counter (Perkin Elmer).

3.6. Apoptosis

For determination of apoptosis by active caspase-3 detection NSCLC cell lines were preincubated for 3 days in normoxia and hypoxia or in serum starved medium with or without 5 g/l BSA (Jackson ImmunoResearch Laboratories) supplementation in normoxia. Thereafter cells were splitted and $5x10^5$ cells were seeded out for each condition. After 4 h for settlement of the cells they were treated. The cells were cisplatin treated for 3 days in medium with FCS or for 3 h in FCS-free medium with subsequent change to normoxic, hypoxic or starvation medium and incubation for 3 days. Other experiments were performed without serum starvation as indicated. For some experiments bevacizumab (Avastin®, Roche, Mannheim, Germany) or human normal immunoglobulin (Nanogam®, Sanquin, Asterdam, Netherlands) as control was combined with cisplatin treatment. Thereafter, cells were harvested by trypsinization and stained with the Caspase-3 Intracellular Activity Assay Kit I (PhiPhiLux® G1D2, Merck, Darmstadt, Germany) according to the manufacturer's protocol with minor changes. Briefly, approximately $5x10^5$ cells were centrifuged and resuspended with 25 µl PhiPhiLux-substrate and 25 µl medium. Samples were incubated for 1 h at 37°C in 5% CO_2 and washed once prior to analysis. Earlier experiments were performed using an Alexa-Fluor-488-conjugated antibody detecting activated caspase-3 (#9669, Cell Signaling Technology, Beverly, MA, USA) according to the manufacturers' protocol. Briefly, cells were trypsinized, pelleted and incubated for 1h with the antibody in the dark. Thereafter, cells were washed once prior to analysis. NSCLC cells with and without caspase-3 activity or positive for activated caspase-3 were distinguished with flow cytometry (FACS Calibur, BD Biosciences, San Jose, USA).

For the TUNEL assay, NSCLC cell lines were preincubated for 3 days in normoxia and hypoxia. Thereafter $1.6x10^4$ cells/well were plated into 8-well chamber slides (Nunc, Langenselbold, Germany). After cisplatin treatment, cells were stained with the In Situ Cell Death Detection Kit (Fluorescein) according to the manufacturer's

protocol (Roche, Mannheim, Germany). Cells were counterstained with a fluorescent mounting medium containing DAPI (Vector Laboratories, Burlingame, USA). Slides were analyzed using an Olympus BX51 microscope at 200-fold magnification and an Olympus DP71 camera. In each well, visual fields were selected randomly and ≥300 cells were counted per sample.

3.7. Western blot analysis

NSCLC cells were harvested with ice-cold RIPA® buffer (Sigma-Aldrich) supplemented with protease inhibitor (Complete mini, Roche). Twenty micrograms of total proteins were separated by 8% or 15% SDS–PAGE and transferred onto nitrocellulose membranes (Bio-Rad, Hercules, CA, USA). The membrane was blocked with 5% skim milk in TBS-T (20 mM Tris–HCl, 140 mM NaCl, 0.1% Tween 20, pH 7.6; 1 h at room temperature), incubated overnight with primary antibodies and for 1 h with specific HRP-conjugated secondary antibody. Immunoreactivity was detected with SuperSignal West Pico Chemiluminescent Substrate (Thermo Scientific, Rockford, IL, USA). Primary antibodies used: mouse monoclonal against HIF-1α, 1:1000 (BD Biosciences, San Diego, CA, USA); rabbit polyclonal against BAX, 1:1000 and BCL-2, 1:500 (Cell Signaling Technology), goat polyclonal against VEGF receptor 1, 2, and 3 (R&D Systems, Minneapolis, USA) and mouse monoclonal against β-actin, 1:3000 (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA).

3.8. RNA isolation and qRT-PCR

Total RNA was extracted using the RNeasy Mini Kit (Qiagen, Hilden, Germany) including DNA digestion by RNase-Free DNase Set (Qiagen) according to the manufactorer´s protocol. cDNA was synthesized using the RevertAid™ H Minus First Strand cDNA Synthesis Kit (Fermentas, St. Leon-Rot, Germany).

Quantitative real-time PCR (qRT-PCR) was performed in triplicates using an ABI Prism 7000 Detection system (Applied Biosystems, Carlsbad, CA, USA). Reaction mix (25 µl): 1x SYBR® Green PCR Master Mix (Invitrogen, Carlsbad, CA, USA), forward and reverse primer (Table S1, 1 mM each), 3 µl cDNA. Beta-actin (ACTB) and hypoxanthine-guanine phosphorybosyltransferase (HPRT1) served as housekeeping genes. cDNA from a cell line (SC-1; human B cell lymphoma cells) known to express all genes of interest, served as calibrator. The results are expressed as relative units based on calculation 2^{-DDCT}, which gives the relative amount of target gene normalized to the endogenous control (geometric mean of the two housekeeping genes) and relative to a normoxic sample. The cycling protocol: One cycle of 50°C for 2 min and 95°C for 10 min followed by 50 cycles consisting of denaturation for 15 s at 95°C, annealing of primers and elongation for 1 min at 60°C.

3.9. ELISA assay

NSCLC cells lines were cultured for 3 days in normoxia and hypoxia in serum starved medium (1% FCS). 4h prior to analysis bevacizumab [50 µg] or human normal immunoglobulin (Nanogam®, Sanquin, Asterdam, Netherlands) as control were added to the medium of the treated group. For analysis a sample of the cell culture supernatant was taken and cells were trypsinized and counted with CASY®. Excretion of VEGF into the culture medium was measured via ELISA (Immuno-Biological Laboratories, Gunma, Japan) according to the manufacturers' instructions. Briefly, samples, blank and standards were pipetted into a 96-well

plate precoated with Anti-Human VEGF (16F1) Mouse IgG monoclonal antibody and incubated for 1h at 37°C. After seven wash steps a HRP conjugated Anti-Human VEGF-1 Rabbit IgG Fab' antibody was added to the wells and incubated for 30 minutes at 4°C. After another nine washing steps the ELISA was developed with chromogen and stop solution. Finally the plate was analysed using a SpectraMax Plus photometer (Molecular Devices, Ismaning, Germany) at 450nm within 30 minutes after adding stop solution and sample values were calculated from the standard curve after subtraction of the blank.

3.10. Immunofluorescence staining

NSCLC cell lines were plated on chamber slides. After one or two days for complete settle down (depending on cell line) the supernatant was discarded and cells were fixed with 4% formalin containing 2% succrose for 10 minutes at room temperature. After two wash steps, cells were permeabilized for 5 minutes with PBS containing 0.5% Triton X-100 (Sigma) at room temperature. After that, cells were washed three times with PBS. Then cells were incubated for one hour with primary antibodies raised against VEGF receptors 1, 2, and 3 (R&D Systems) or a goat-IgG isotype antibody as control (Santa Cruz) in PBS containing 1% BSA (Jackson ImmunoResearch Laboratories, West Grove, USA) at room temperature. Three times washing with PBS followed. Finally cells were incubated for an hour in the dark at room temperature with an anti-goat Alexa-Flour-488 labelled secondary antibody (Invitrogen) and washed thrice, before covered with an fluorescence mounting medium containing DAPI (Vectashield®, Vector Laboratories, Burlingame, USA). Images were taken using an Olympus BX51 microscope at 200-fold magnification and an Olympus DP71 camera.

3.11. Statistical analysis

Each experiment was repeated at least three times. The data were compiled and analyzed with the software package GraphPad Prism, version 5.03 (La Jolla, CA). Group differences were calculated using Student´s t-test, one- or two-way ANOVA

with post-hoc analysis, or Kruskal-Wallis test followed by Mann-Whitney-U tests and Bonferroni correction as applicable. $p<0.05$ was considered significant.

4. RESULTS

4.1. Effects of hypoxia on proliferation and viability in human NSCLC cells

To analyze the influence of hypoxia (1% O_2) on proliferation and viability of NSCLC cells, A549 and NCI-H358 cells were exposed to hypoxia for up to 96 h. Cells were investigated every 24 h with electronic pulse area analysis. A remarkable reduction of growth rate was found in hypoxic A549 cells (Fig. 8A). NCI-H358 cells exhibited a strongly reduced growth rate compared to A549 cells in normoxia and their growth rate was not significantly reduced in hypoxia (Fig. 8A). Hypoxia-induced growth reduction resulted in lowered cell numbers after 96 h (-56±5%) in A549 ($p<0.001$) and (-35±12%) in NCI-H358 cells ($p>0.05$), respectively. It is important to note that this difference in the total cell number could not be explained by an increased cell death because the viability of NSCLC cells was not diminished by up to 96 h continuous hypoxia (Fig. 8B). This is also visible in phase-contrast images after three days incubation in normoxia and hypoxia (Fig.9).

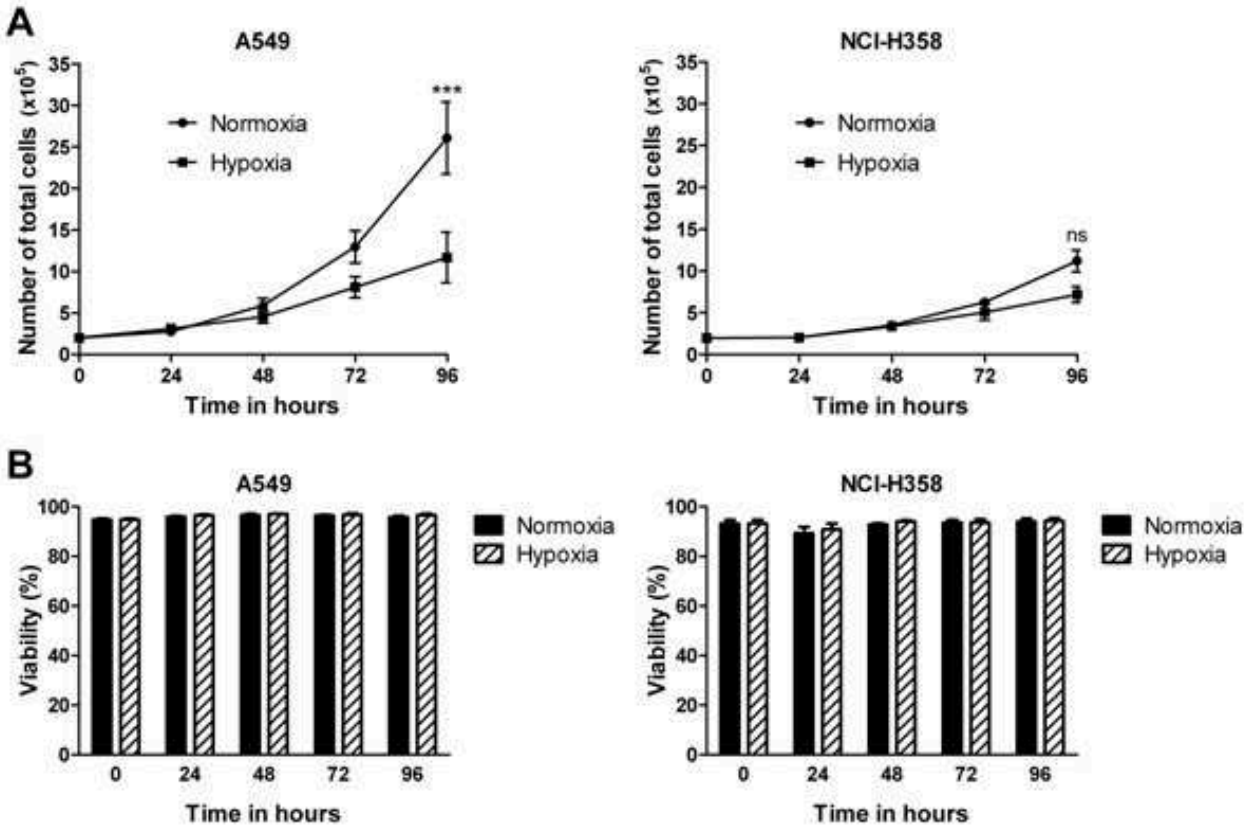


Fig. 8. Hypoxia inhibits proliferation of NSCLC cells without influencing viability. (A) A549 and NCI-H358 cells were incubated in normoxia or hypoxia (1% O2) for up to 96 h. For each time point cells were harvested including cells from supernatant. The number of total cells was measured with the electronic pulse area analysis technique (CASY® cell counter). (B) Viability of the cells was assessed in the same experiment with CASY®. Mean values +/- SD are shown. ***, $p<0.001$; ns, not significant, on Bonferroni post-hoc analysis after two-way ANOVA.

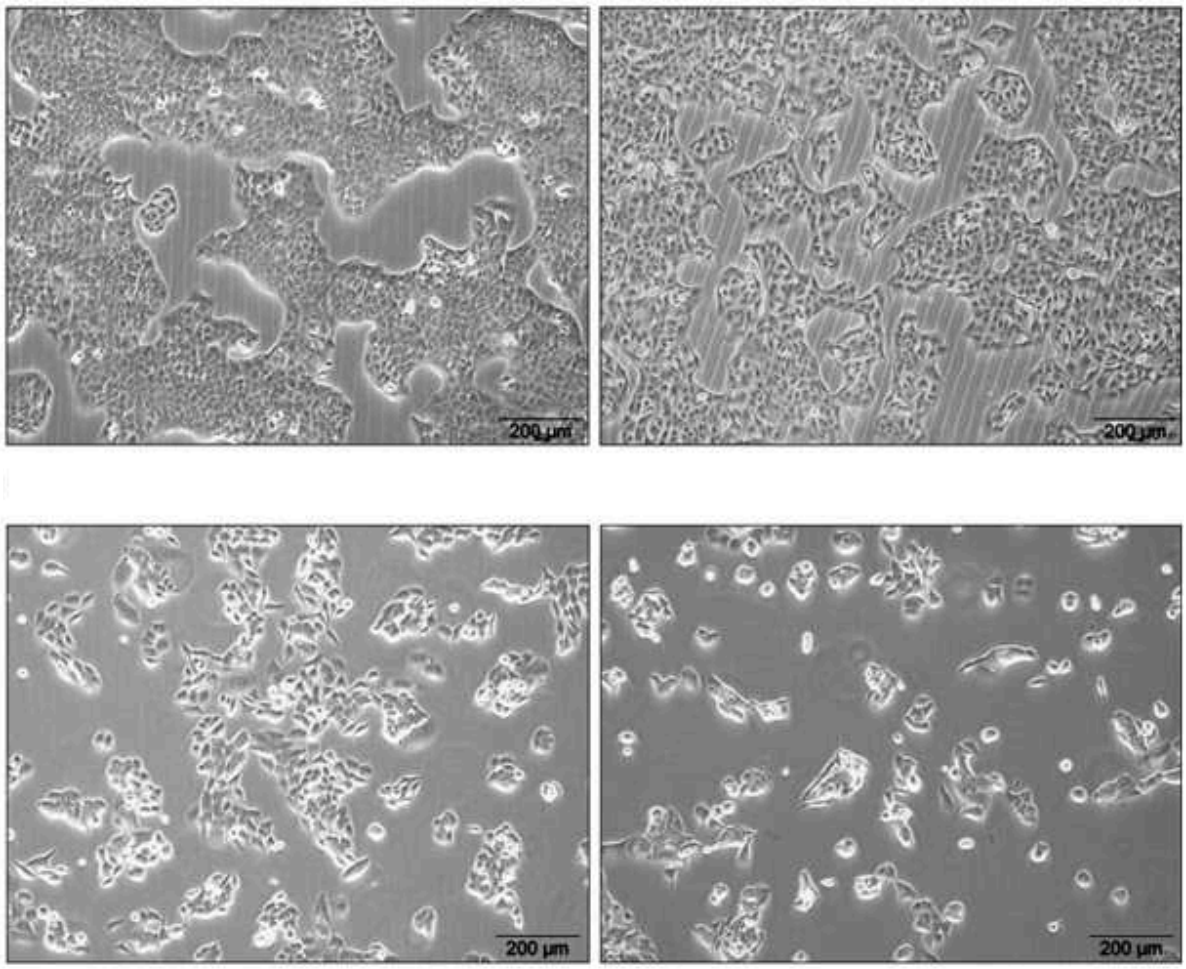


Fig. 9. Hypoxia does not induce cell death in NSCLC cells. Phase contrast images of (C) A549 and (D) NCI-H358 cells after 3 days in normoxia (left) and hypoxia (right).

4.2. Effects of hypoxia on cisplatin-induced cytotoxicity

To investigate the effect of hypoxia (1% O_2) on cisplatin-induced cell death, electronic pulse area analysis for viability was performed after treatment of normoxic and chronic hypoxic cells for 72 h with different concentrations of cisplatin in normoxia and hypoxia. Viability of A549 cells decreased with increasing cisplatin dose ($p<0.001$). A significantly diminished cytotoxic effect of cisplatin in hypoxia was found ($p<0.01$; Fig. 10A). This was most pronounced with the highest cisplatin concentration (ANOVA AxB $p<0.05$). The effects on NCI-H358 cells were very similar (Fig. 10B).

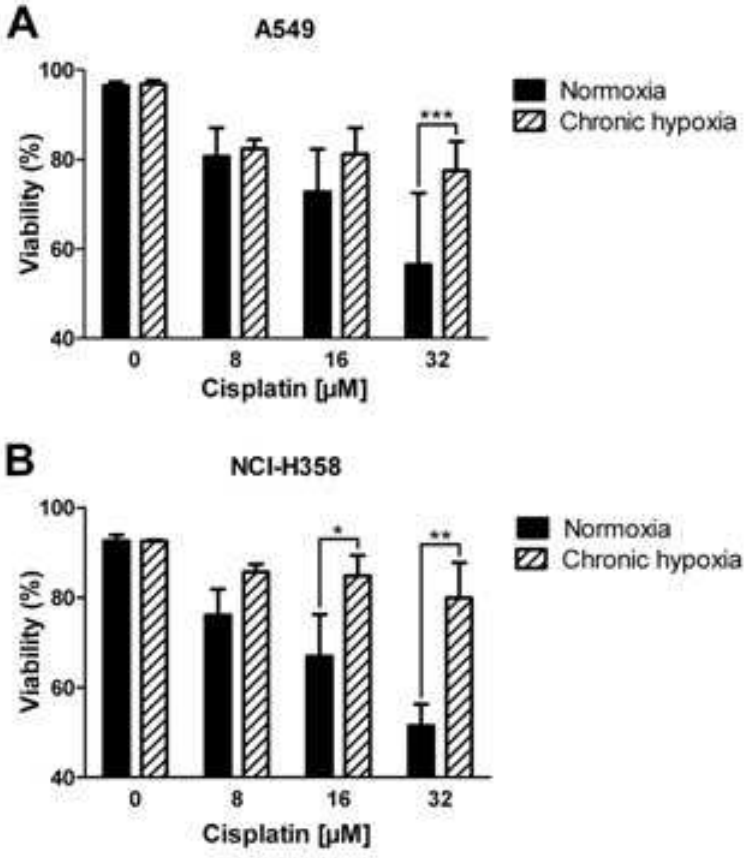


Fig. 10. Hypoxia reduces cisplatin cytotoxicity in NSCLC cell lines. Normoxic and chronic hypoxic A549 (A) and NCI-H358 (B) cells were incubated for 3 days with cisplatin at different concentrations. The viability of the cells was measured using CASY® cell counter. Mean values +/- SD are shown. *, $p<0.05$; **, $p<0.01$ and ***, $p<0.001$, on Bonferroni post-hoc analysis after two-way ANOVA.

4.3. Effects of hypoxia on cisplatin-induced apoptosis

As cisplatin is known to cause cell death via apoptosis, we treated normoxic and chronic hypoxic cells with different concentrations of cisplatin. To find the optimal experimental conditions we performed time-course experiments (Fig. 11). A reliable effect was seen at 72 hours. The best cisplatin concentration was titrated and 32 µM cisplatin was used for further experiments (TUNEL-assay, Fig. 12A and B and Fig. 13A). Cisplatin induced concentration-dependent apoptosis in both cell lines ($p<0.001$, respectively). Hypoxia almost completely abolished this effect in both cell lines ($p<0.001$, respectively). To confirm these results, apoptosis was additionally investigated with an advanced technique namely the PhiPhiLux® assay. After 72 h of treatment with 32 µM cisplatin, a significant inhibition of apoptosis was found in chronic hypoxic cells compared to normoxic cells (A549 $p<0.001$, NCI-H358 $p<0.01$; Fig. 12C and 13B), confirming the TUNEL and activated capase-3 staining data. There was no significant apoptosis in untreated normoxic or chronic hypoxic cells.

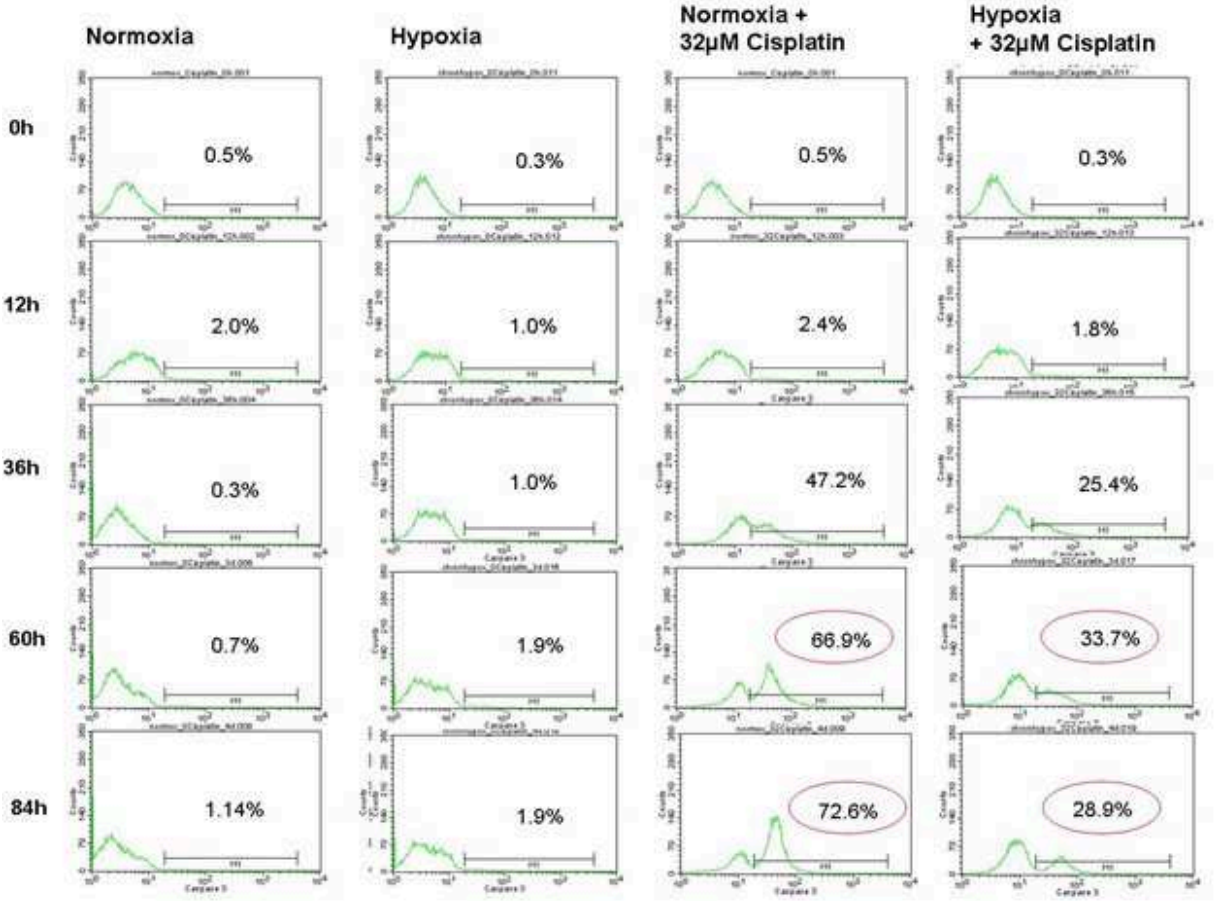


Fig. 11. Hypoxia reduces cisplatin cytotoxicity by inhibition of apoptosis. Normoxic and hypoxic A549 cells were incubated with 32 µM cisplatin for up to 84 h. Raw data images of flow cytometric analysis of activated caspase-3 staining. Numbers depict the percentage of cells with activated caspase-3.

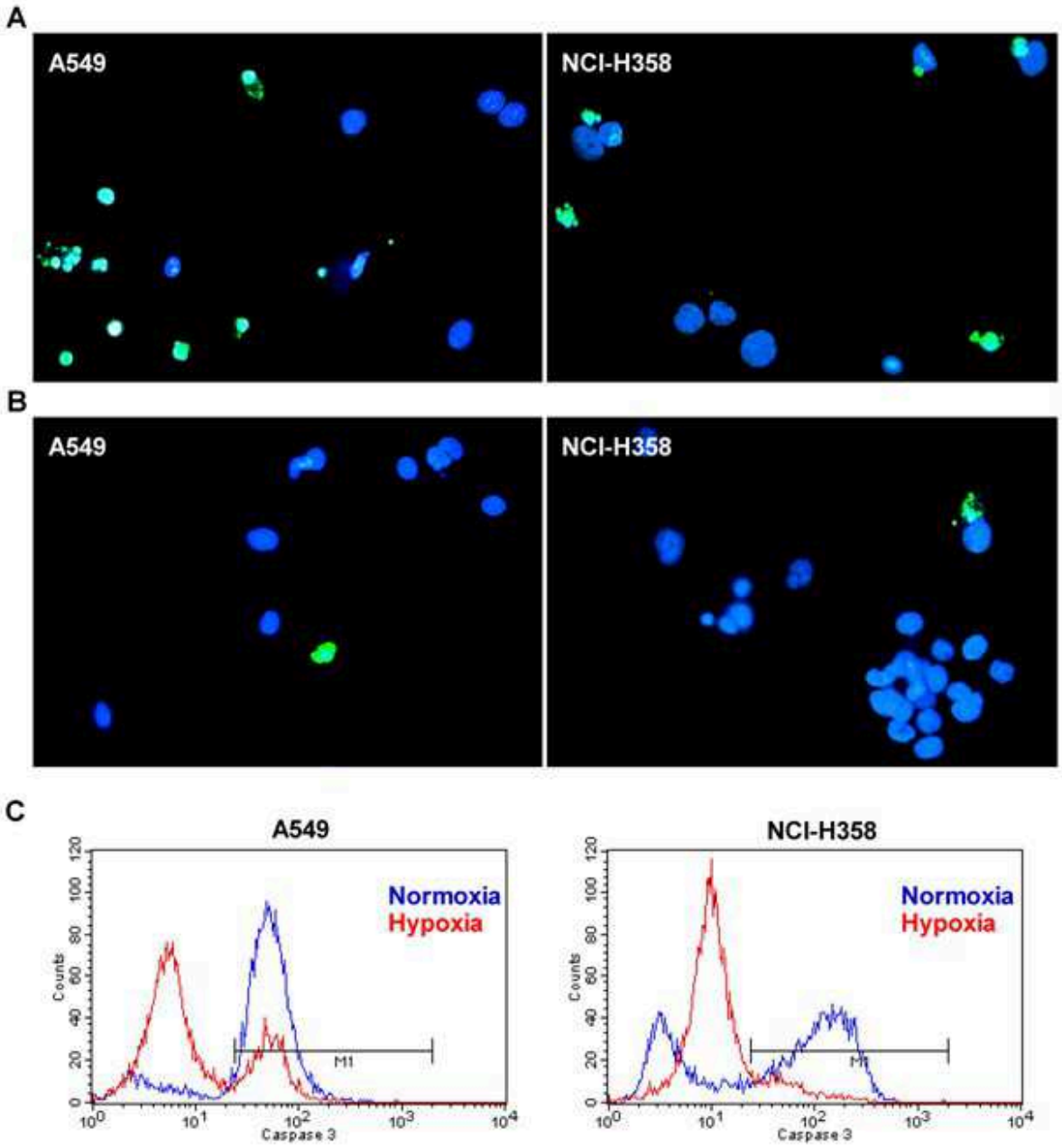


Fig. 12. Hypoxia reduces cisplatin cytotoxicity by inhibition of apoptosis. Normoxic and chronic hypoxic A549 and NCI-H358 cells were incubated with 32 µM cisplatin for 72 h. Representative images of a TUNEL assay in normoxia (A) and hypoxia (B). Representative histograms of the flow cytometric analysis of cells with caspase-3 activity (PhiPhiLux®)(C).

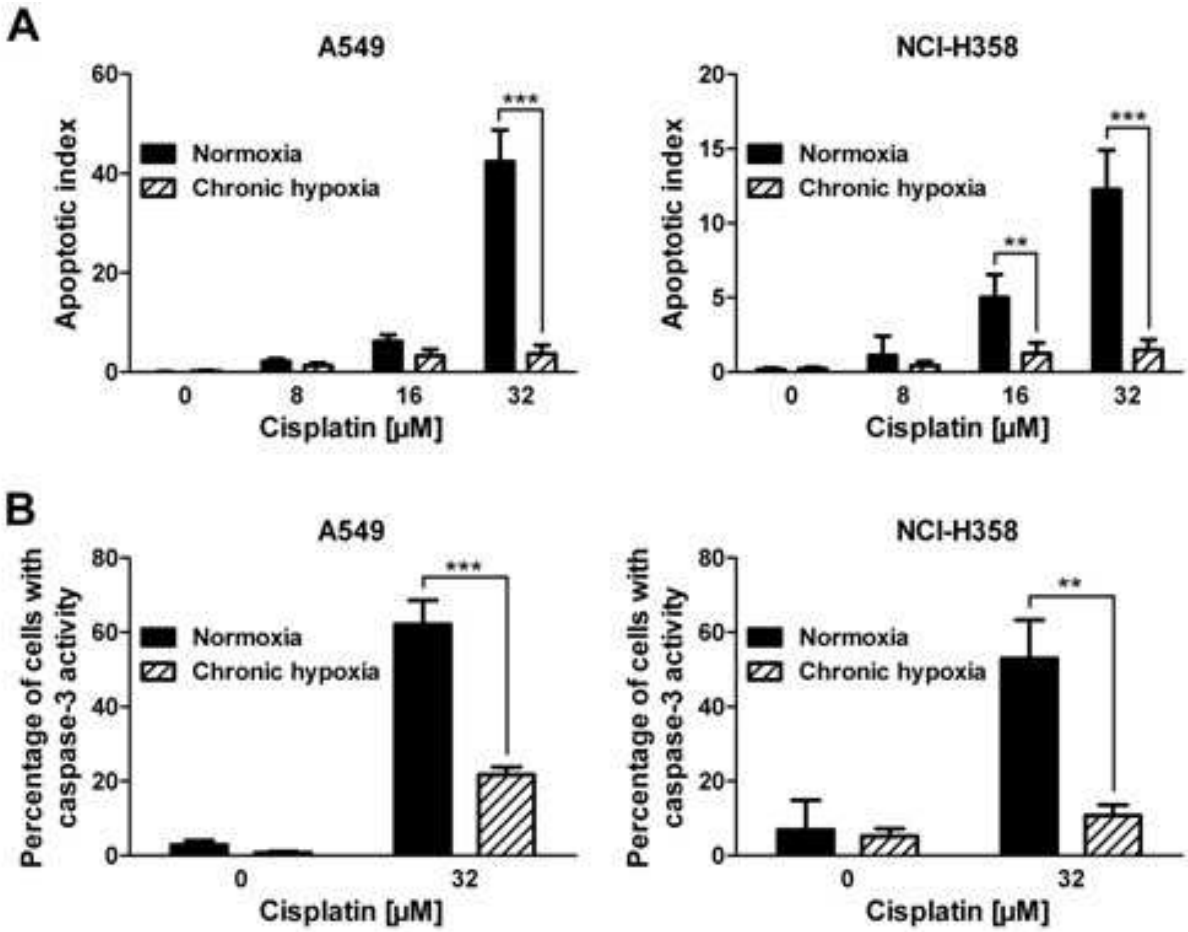


Fig. 13. Statistical analysis of cisplatin-induced apoptosis. Three independent experiments of TUNEL (A) and PhiPhiLux® (B) were evaluated. Mean values +/- SD are shown. **, $p<0.01$ and ***, $p<0.001$, on Bonferroni post-hoc analysis after two-way ANOVA. Student´s t-test was used for comparison of apoptosis rates of treated normoxic and hypoxic cells assessed by PhiPhiLux® assay.

4.4. Effects of re-oxygenation on hypoxia-induced cisplatin resistance

Chronic hypoxic A549 cells were transferred to normoxia and incubated for additional 1, 2, or 3 days and then treated with 32 µM cisplatin. Chronic hypoxic cells without re-oxygenation and normoxic cells served as reference (Fig. 14A). Apoptosis induction by cisplatin was significantly inhibited by hypoxia ($p<0.05$; Fig. 14B) and completely restored after 3 days of re-oxygenation ($p<0.05$; Fig. 14B)

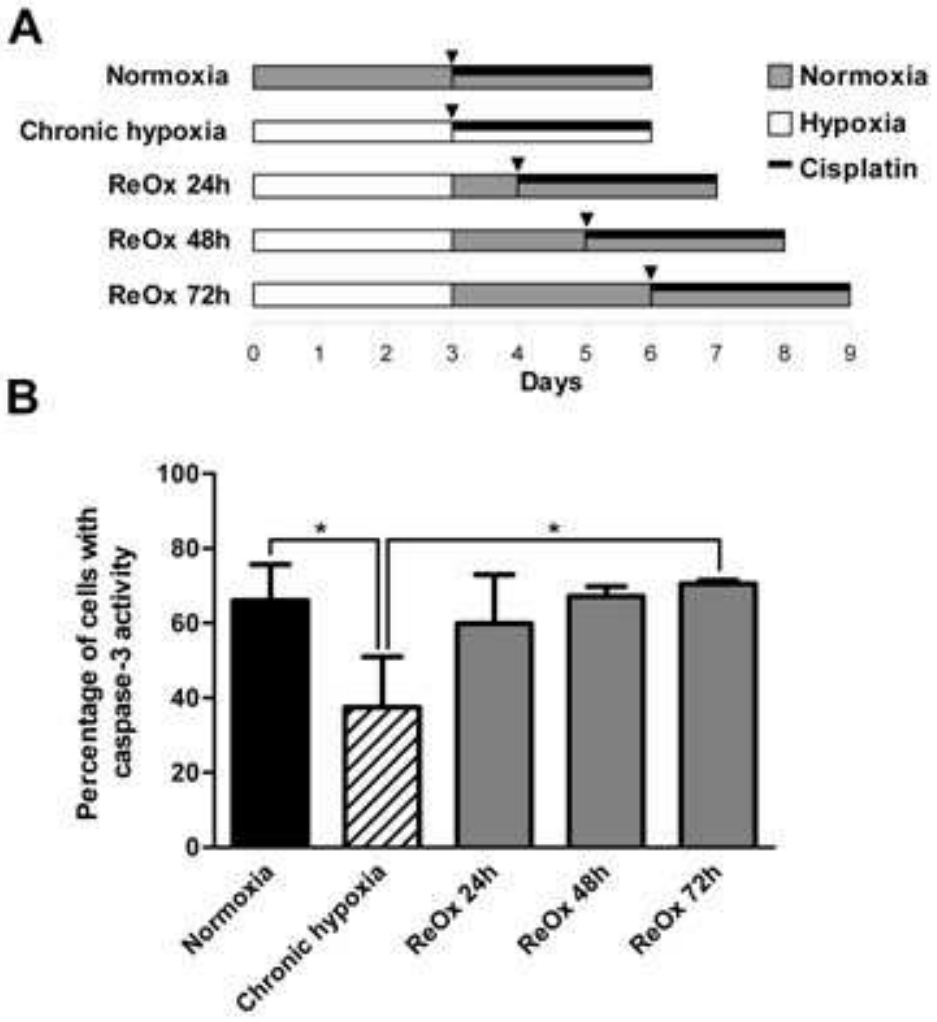


Fig. 14. Cisplatin resistance in hypoxic A549 cells is reversible after re-oxygenation. (A) Scheme of the experimental setup: chronic hypoxic A549 cells were transferred back into normoxia and incubated in normoxia for an additional 24, 48 and 72 h period and then treated with 32 µM cisplatin for another 72 h. Treated chronic hypoxic cells without re-oxygenation and normoxic cells served as reference. (B) Cells with caspase-3 activity were assessed with the PhiPhiLux® assay and flow cytometry. Mean values +/- SD are shown. One-way ANOVA $p<0.05$. *, $p<0.05$, on Tukey post-hoc analysis. ReOx: Cells reoxygenated for a predefined period after 3 days of hypoxia.

4.5. Sensitivity to cisplatin is largely independent from growth rate of A549 cells

Cisplatin-induced apoptosis in NSCLC cells might be dependent on the growth rate. We therefore adjusted the growth rate of normoxic A549 cells to the level of hypoxic A549 cell growth by means of serum starvation. We accomplished this with 1% FCS as evidenced by cell counting with CASY® and [^{3}H]-thymidine uptake assay (Fig. 15A and B). Although the growth rate was largely inhibited by serum starvation in normoxia, apoptosis induction by 32 µM cisplatin after 72 h was equal in serum starved A549 cells compared to non-starved A549 cells (Fig. 15B and C). Similar results were obtained using TUNEL assay (Fig. 15D). As cisplatin is bound to proteins in FCS, a reduction of serum in the culture medium might lead to increased free cisplatin and thereby mask the influence of growth reduction in serum starved cells. To exclude this, we supplemented the starvation medium with BSA to the protein level of medium containing 10% FCS (5 g/l). Adding BSA to the starved cells had no influence on apoptosis induction or growth rate. (Fig. 15B and C). To confirm this data we performed a short-term cisplatin treatment (3 h) in medium without FCS (Fig. 15E).

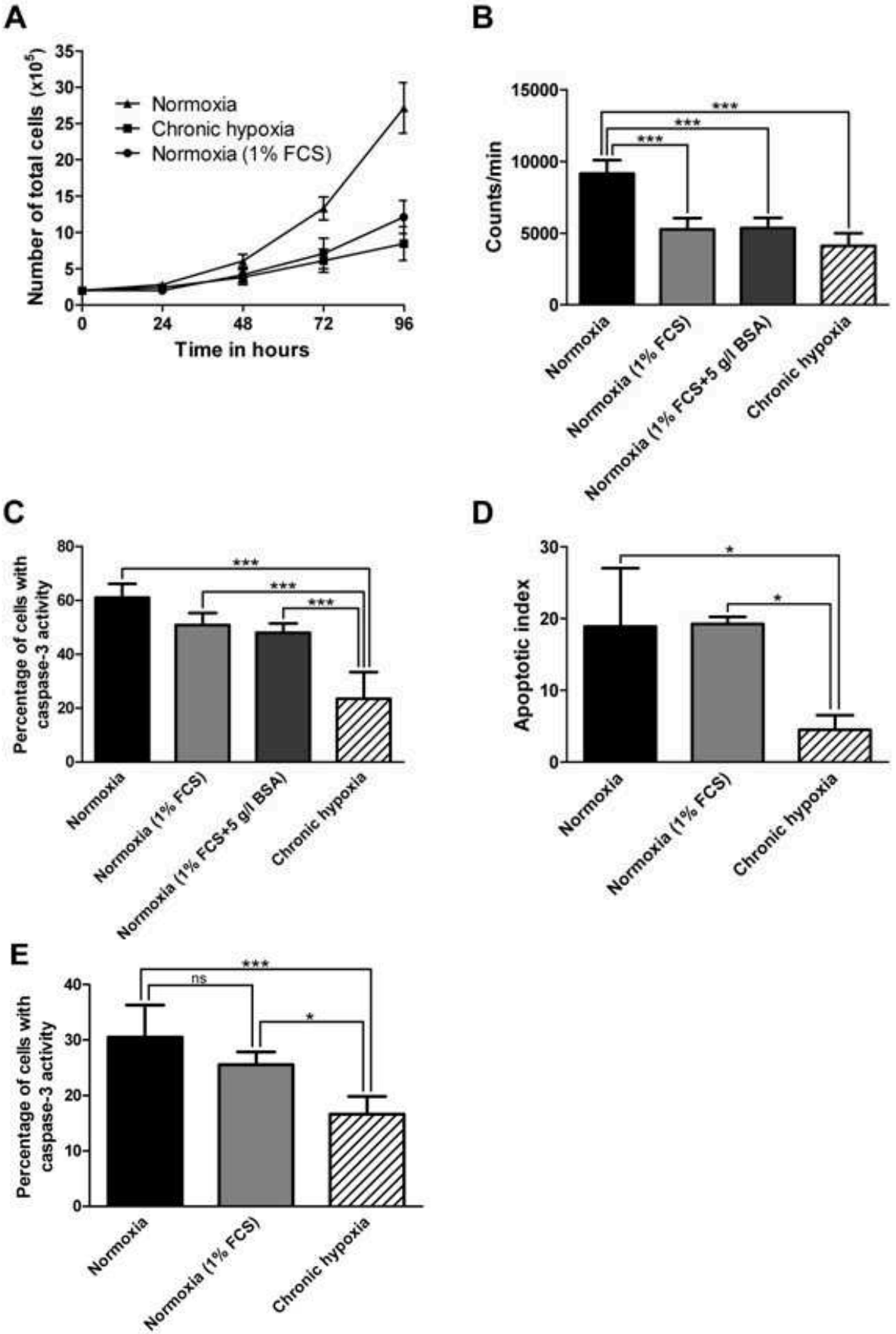

A
Number of total cells (x10^5)
35
30
25
20
15
10
5
0
Normoxia
Chronic hypoxia
Normoxia (1% FCS)
0
24
48
72
96
Time in hours
B
Counts/min
15000
10000
5000
0
***
***
***
Normoxia
Normoxia (1% FCS)
Normoxia (1% FCS+5 g/l BSA)
Chronic hypoxia
C
Percentage of cells with caspase-3 activity
80
60
40
20
0
***
***
***
Normoxia
Normoxia (1% FCS)
Normoxia (1% FCS+5 g/l BSA)
Chronic hypoxia
D
Apoptotic index
30
20
10
0
*
*
Normoxia
Normoxia (1% FCS)
Chronic hypoxia
E
Percentage of cells with caspase-3 activity
40
30
20
10
0
***
ns
*
Normoxia
Normoxia (1% FCS)
Chronic hypoxia

Fig. 15. Hypoxia-induced apoptosis resistance to cisplatin is largely independent from growth rate. (A) Growth rate of A549 cells in hypoxic condition was mimicked by serum starvation (1% FCS) of cells in normoxia (electronic pulse area analysis). (B) Thymidin-uptake assay after 72 h; ANOVA $p<0.001$, no significant difference between starvation, starvation + BSA and chronic hypoxia. (C) Percentage of cells with caspase-3 activity 72 h after cisplatin [32 µM] in normoxia, with and without serum starvation (+/- 5 g/l BSA) and in chronic hypoxia; ANOVA $p<0.001$, no significant difference between normoxia, starvation and starvation + BSA. (D) Same analysis for TUNEL assay; ANOVA $p<0.05$. (E) Percentage of cells with caspase-3 activity after 3 h cisplatin [32 µM] in FCS-free medium and subsequent change to normoxic, hypoxic or starvation medium and incubation for 3 days; ANOVA $p<0.001$. Mean values +/- SD are shown. ns, not significant, *, $p<0.05$ and ***, $p<0.001$, on Tukey post-hoc analysis. FCS: Fetal calf serum; BSA: Bovine serum albumin.

4.6. Regulation of pro- and anti-apoptotic proteins in hypoxia

In A549 and NCI-H358 cells we found a time-dependent upregulation of HIF-1α in hypoxia (Fig. 17A). As NSCLC cells develop resistance to apoptosis in hypoxia, changes in proteins regulating apoptosis are to be expected. Indeed, by screening the mRNA level of a panel of BCL-2-family proteins from normoxic, starved and hypoxic NSCLC cells we found a significant down-regulation of the pro-apoptotic BCL-2-associated X protein (BAX) in hypoxic A549 cells compared to normoxic cells (Fig. 16). There were no significant changes comparing starved and normoxic cells (Fig. 16). In this screening, members of the pro-apoptotic subfamilies “BH3” (PUMA, NOXA, BMF, BIM, BAD, BID and BIK) and “BAX” (BAX), as well as members of the anti-apoptotic subfamily “BCL-2” (BCL-2, BCL-XL and MCF-1) were included. BAD, BID and BIK were finally excluded from analysis due to very low overall expression. In NCI-H358 cells we found no significant changes of pro- or anti-apoptotic proteins (data not shown). These results were confirmed on the protein level for BAX and the corresponding anti-apoptotic protein BCL-2 (Fig. 17B and C).

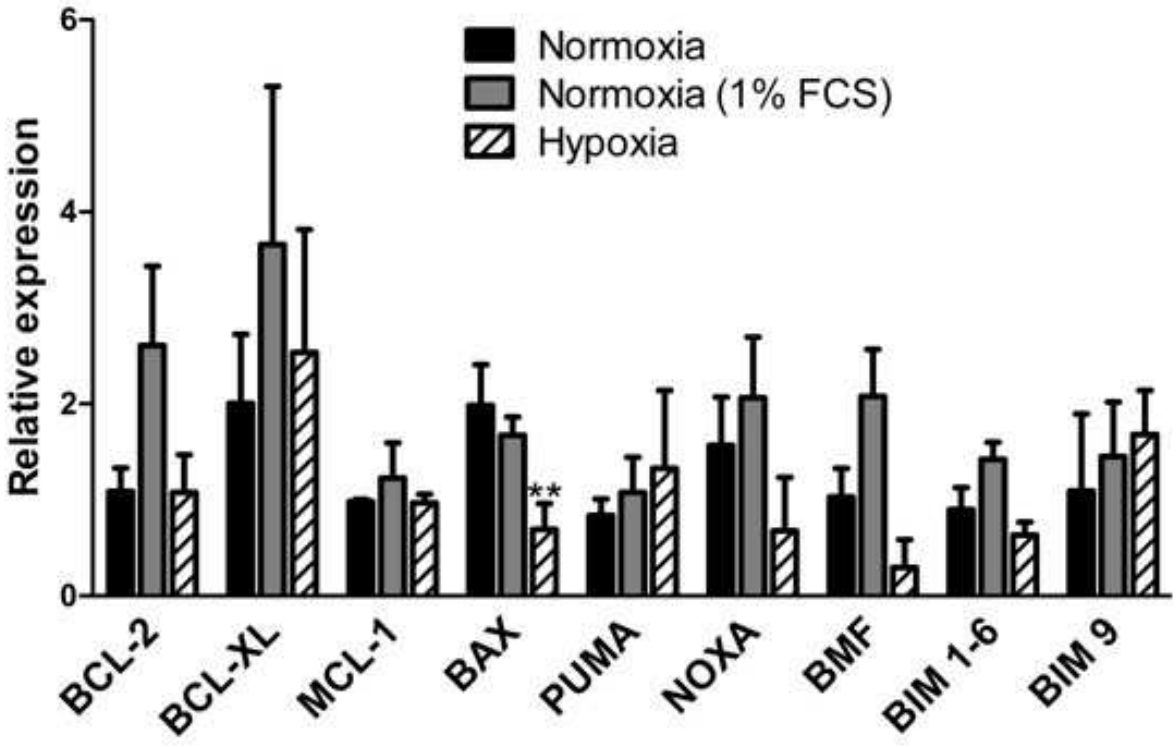


Fig. 16. Regulation of pro- and anti-apoptotic proteins on the mRNA level. A549 cells were incubated for 3 days in normoxia, with and without serum starvation and hypoxia. Thereafter target genes were analyzed with quantitative RT-PCR. Mean values +/- SD are shown. **, $p<0.01$, on Mann-Whitney-U tests after Bonferroni correction. BCL-2: B-cell lymphoma 2; BCL-XL: B-cell lymphoma-extra large; MCL-1: Induced myeloid leukemia cell differentiation protein; BAX: BCL-2–associated X protein; PUMA: p53 upregulated modulator of apoptosis; BMF: BCL-2-modifying factor; BIM: BCL-2-like protein 11 (Only 3 out of 9 transcription variants of BIM are leading to proteins, these are variant 1, 6 and 9. Two primer pairs were needed to cover all 3 variants). BAD, BID and BIK were excluded from analysis due to very low overall expression. In cooperation with Mag.rer.nat. Dr.scient.med. Alexander Deutsch, who performed the qPCR and statistics for this experiment.

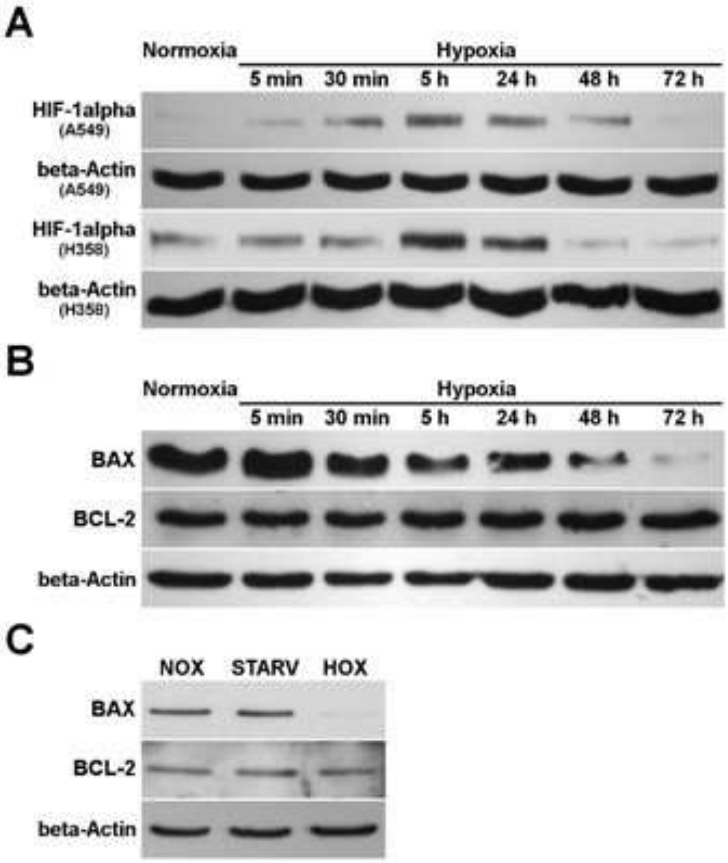


Fig. 17. Intracellular levels of HIF-1α and apoptosis proteins. (A) A549 and NCI-H358 cells were incubated for 3 days in normoxia and then placed for indicated time periods in hypoxia. Thereafter cells were subjected to Western blot analysis to determine the intracellular levels of HIF-1α. (B) Western blot analysis of BAX and BCL-2 in A549 cells under hypoxic conditions. (C) A549 cells were incubated for 3 days in normoxia, with and without serum starvation and hypoxia. Thereafter cells were subjected to Western blot analysis to determine the intracellular levels of BAX and BCL-2. NOX: Normoxia; STARV: Starvation; HOX: Hypoxia.

4.7. NSCLC cells secrete VEGF

The prerequisites of an autocrine loop are that the same cell not only secrets the ligand but also expresses the receptor for this ligand. Our question was if NSCLC cell lines produce and also secrete VEGF and if this is relevant for apoptosis and proliferation. To answer these questions we utilized an ELISA assay able to detect human VEGF. Cells were cultured for three days in normoxia and hypoxia. Thereafter, the supernatant was analyzed and the cell count determined to normalize the VEGF content. Five out of six NSCLC cell lines secreted VEGF to a different degree. However, the amount of secreted VEGF was higher in hypoxia for all cell lines secreting VEGF (Fig. 18). In the same experiments, we tested if the already clinically approved anti-VEGF antibody bevacizumab (Avastin®) is able to scavenge the VEGF secreted by the NSCLC cell lines under cell culture conditions. Therefore 4h prior to analysis bevacizumab or control IgG (Nanogam®) were added to the culture medium in normoxia. Bevacizumab almost completely blocked the binding sites of VEGF, making it undetectable for the ELISA antibodies, whereas the control IgG did not interfere (Fig. 18)

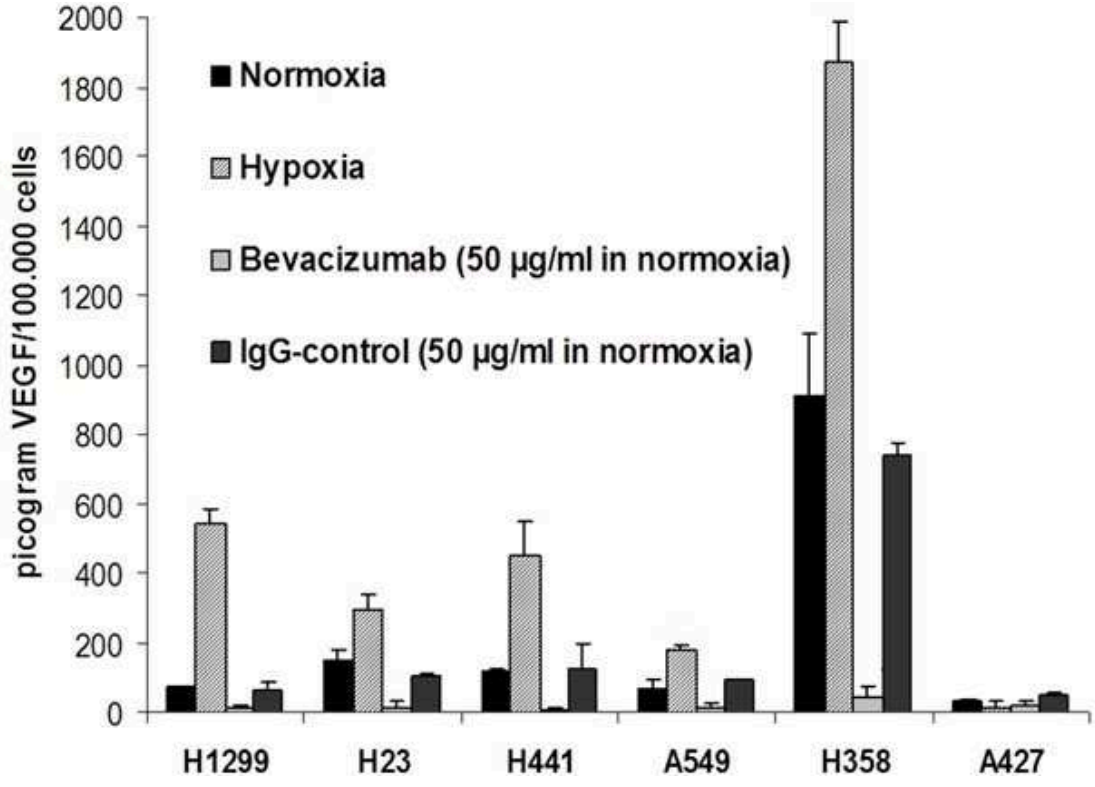


Fig. 18. Secretion of VEGF by NSCLC cell lines. NSCLC cell lines were cultured for 3 days in normoxia and hypoxia. 4h prior to analysis, bevacizumab or control were added to the medium. For analysis a sample of the cell culture supernatant was taken and cells were trypsinized and counted with CASY®. The supernatant was subjected to a VEGF-ELISA and the results normalized to the cell count. Mean values +/- SD are shown. *, $p<0.05$; **, $p<0.01$ and ***, $p<0.001$, on Tukey post-hoc analysis after ANOVA for each cell line.

4.8. NSCLC cells express VEGF receptors

In order to sense VEGF NSCLC cells also have to express the VEGF receptors. Using Western blot we could determine VEGF receptor 1 in all cell lines, while receptor 2 was hardly expressed (Fig. 19). The same result was obtained with immunofluorecent staining (Fig. 20).

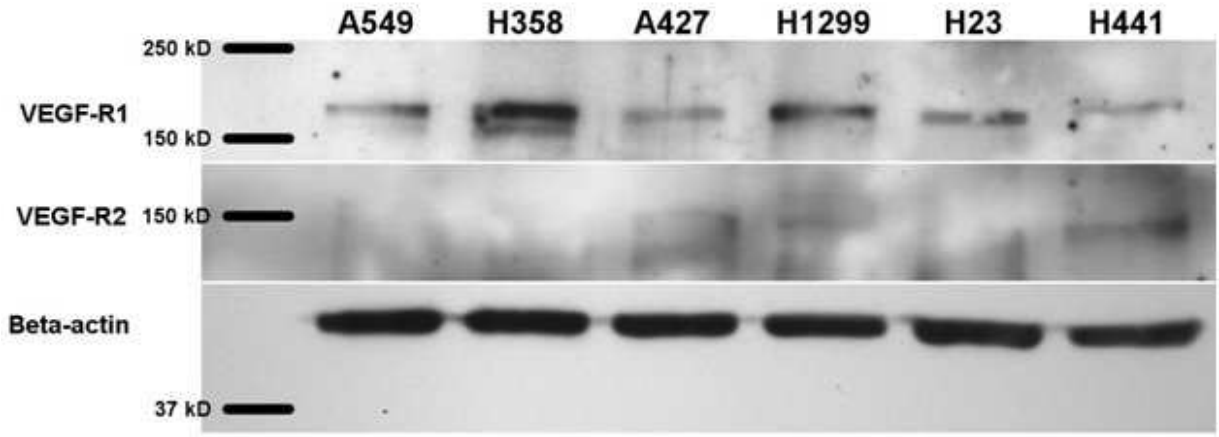


Fig. 19. Expression of VEGF receptors in NSCLC cell lines. Normoxic cells were subjected to Western blot analysis to determine the expression of VEGF receptor 1 and 2. Beta-actin served as loading control.

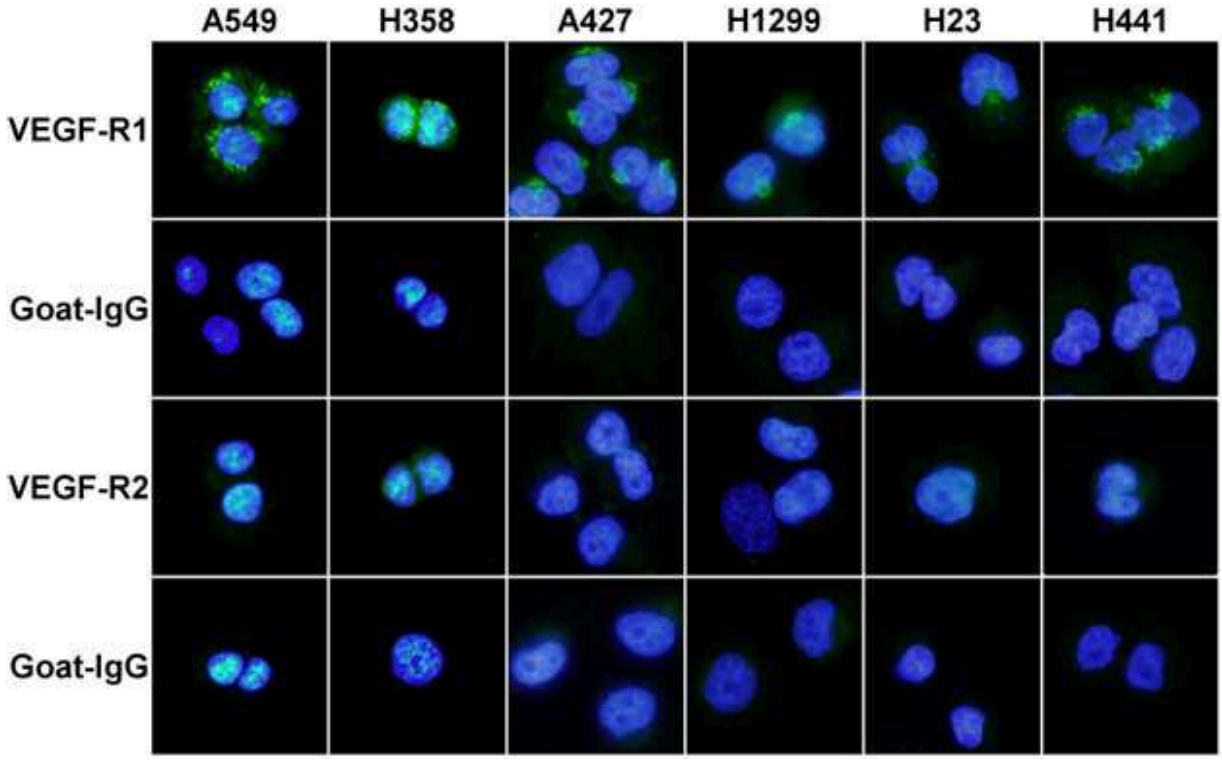


Fig. 20. Expression of VEGF receptors in NSCLC cell lines. NSCLC cell lines were plated on chamber slides and then subjected to an immunofluorescence staining detecting VEGF receptor 1 and 2 (green). A goat-IgG isotype antibody served as control. DAPI counterstaining (blue).

4.9. Influence of VEGF on proliferation in NSCLC cells

Having the prerequisites fulfilled, the next step was to proof if the autocrine loop of VEGF in fact is functional in NSCLC cell lines. First VEGF binding to its receptor was blocked with bevacizumab (Fig. 21). Neither in normoxia nor in hypoxia proliferation could be inhibited. To investigate if VEGF receptors was functional cells were stimulated with human VEGF and thymidin-uptake assay was performed subsequently (Fig. 22). Out of the six cell lines only one responded significantly and dose-dependently to the VEGF stimulation ($p=0.035$). Summing up the response of all cell lines revealed no significant response to VEGF stimulation (Fig. 22 top).

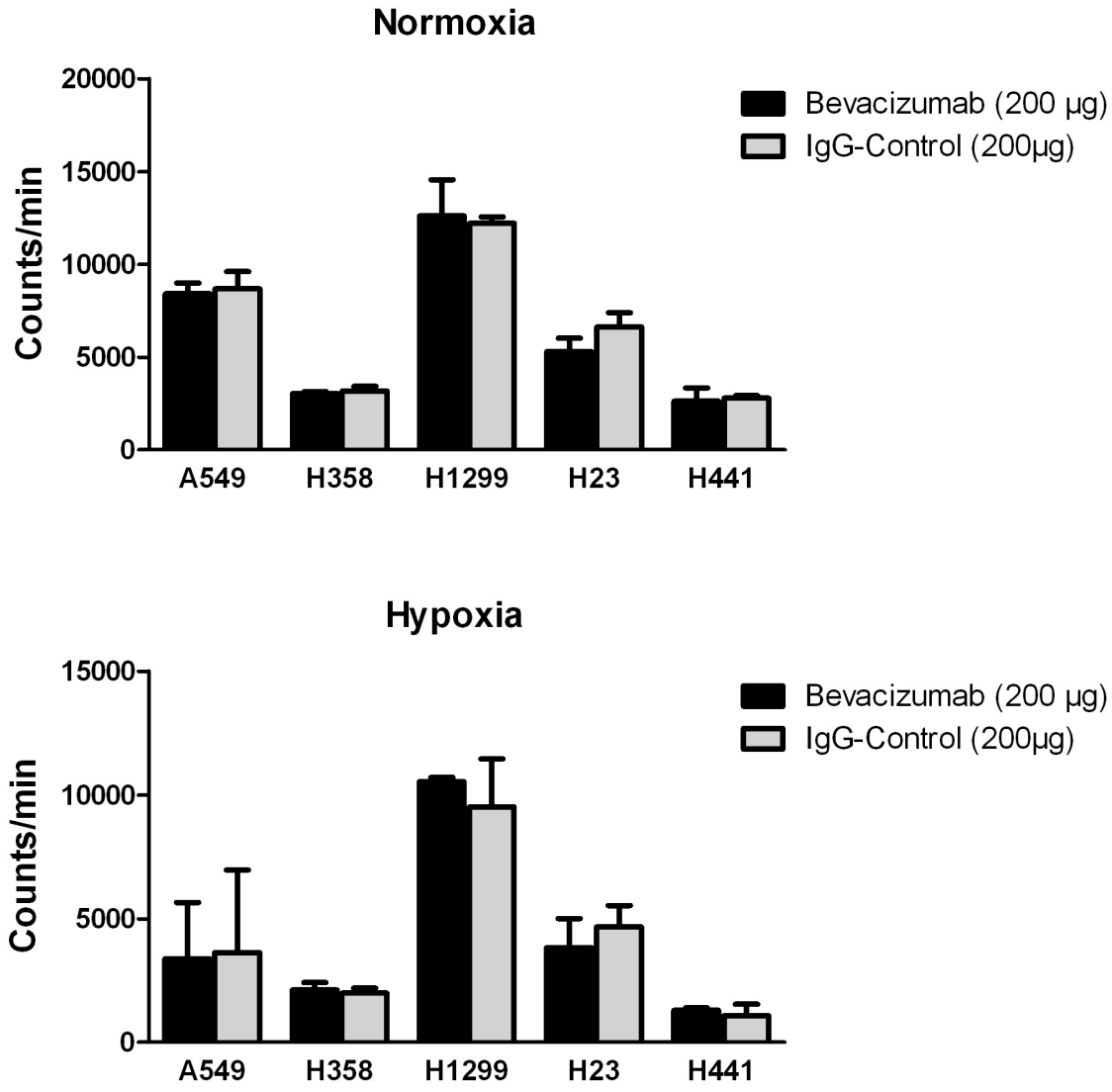


Fig. 21. Effect of bevacizumab on NSCLC proliferation in normoxia and hypoxia. Thymidin uptake assay after 72h treatment with bevacizumab (200µg) or IgG-control in 10% FCS medium. Mean values +/- SD are shown. Statistical analysis with student´s t-test revealed no significant difference between treatment and control for each cell line.

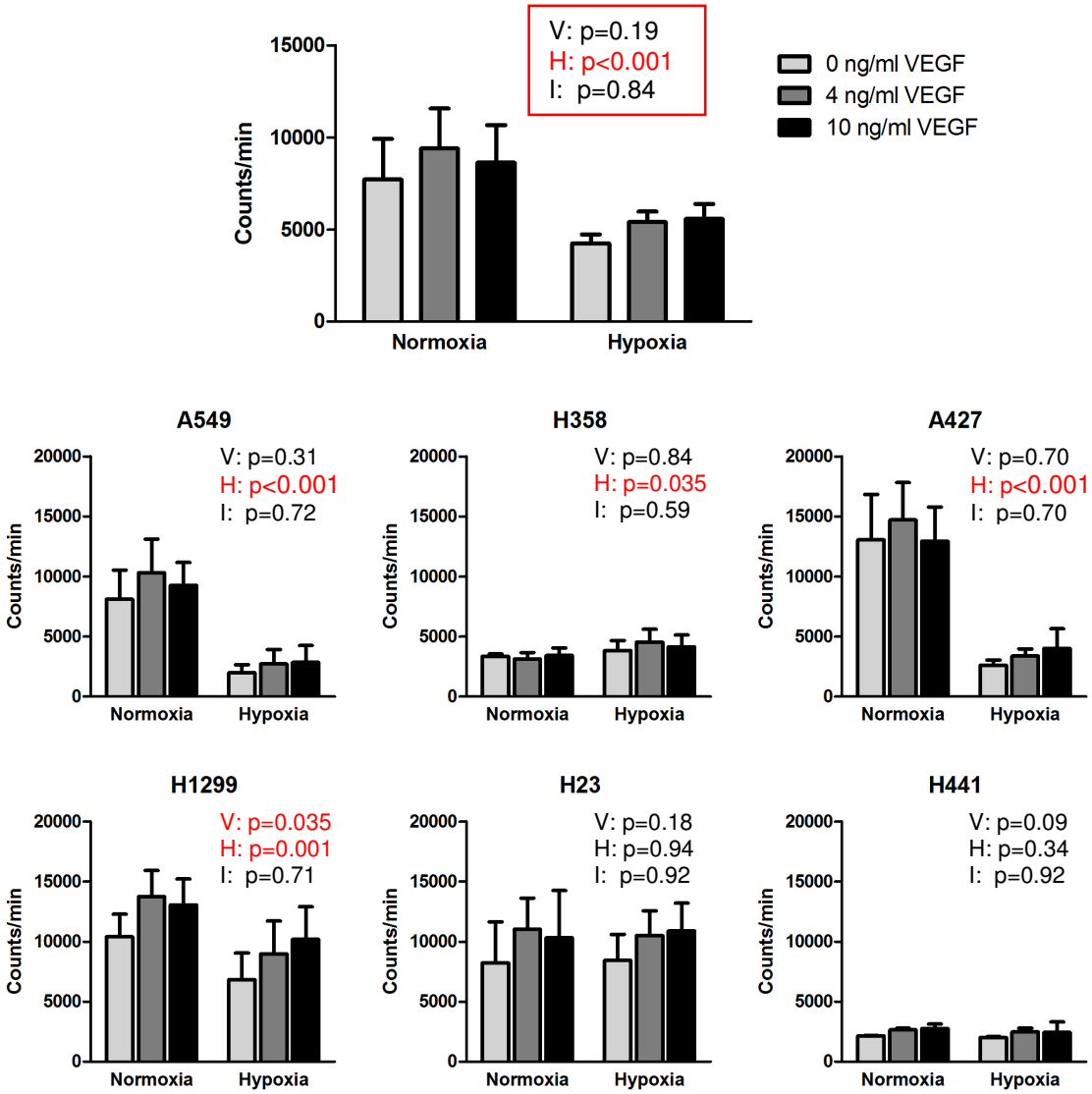


Fig. 22. Effect of VEGF stimulation on NSCLC proliferation. Thymidin uptake assay after 72h treatment with predefined concentrations of VEGF in 0.5% FCS medium. Top figure resembles the aggregate of all cell lines. Mean values +/- SD are shown. Tukey post-hoc analysis after two-way ANOVA. V=VEGF, H=Hypoxia, I=Interaction.

4.10. Influence of VEGF on apoptosis in NSCLC cells

The second hypothesis was that blocking the VEGF pathway with bevacizumab would induce apoptosis in NSCLC cell lines. Therefore, cells were incubated with bevacizumab (250 µg/ml) or control (Nanogam®) for 3 days and then apoptosis was assessed with PhiPhiLux® (Fig. 23). The second approach was to investigate if bevacizumab would enhance the apoptosis-induction of cisplatin. Thus, cells were coincubated for 3 days with cisplatin (32 µM) and bevacizumab (250 µg/ml) or control (Fig. 24). Neither significant induction nor enhancement of apoptosis by bevacizumab occurred.

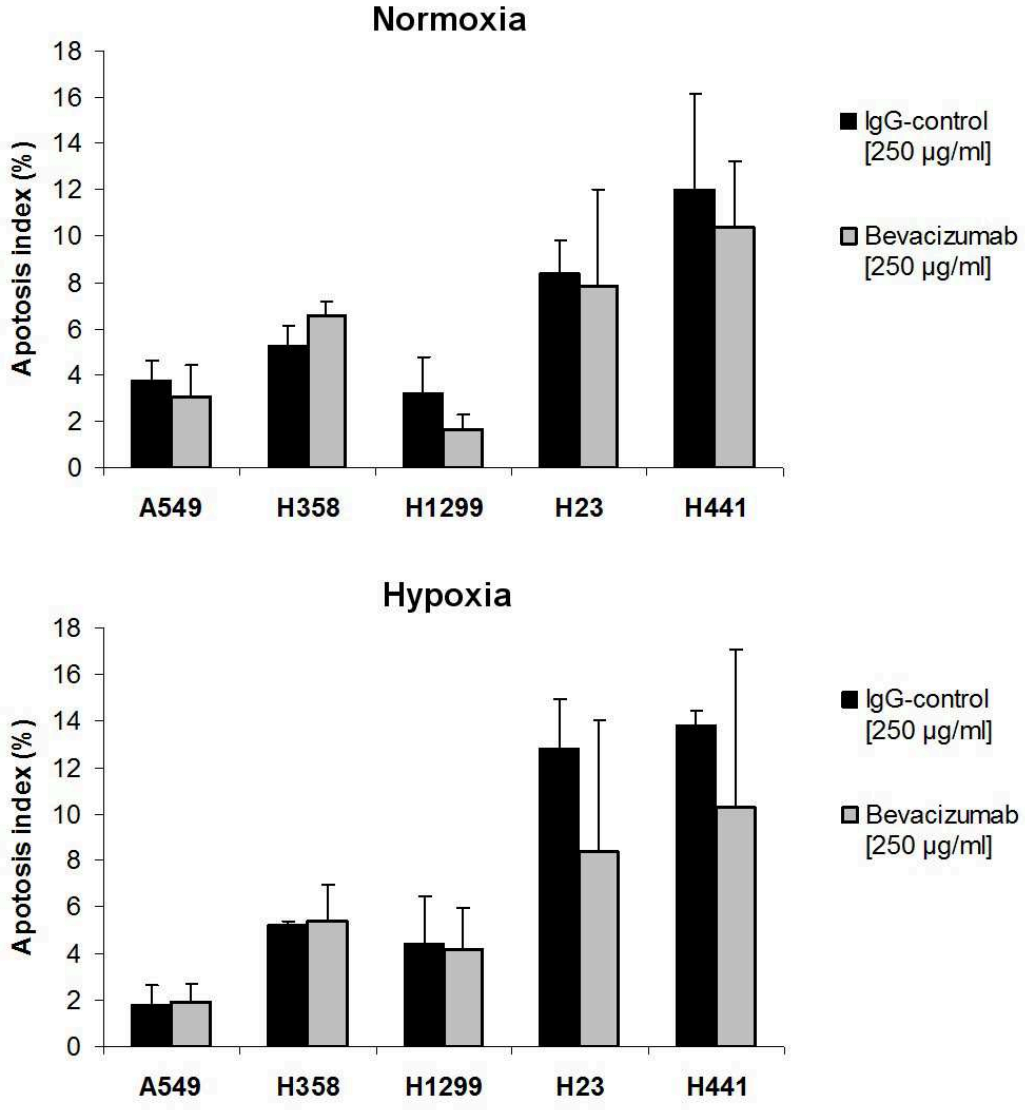


Fig. 23. Apoptosis induction by bevacizumab. Normoxic and hypoxic NSCLC cell lines were treated for 3 days with bevacizumab [250 µg/ml] or control. Thereafter cells were stained with PhiPhiLux® for active caspase-3 and analysed with flow cytometry. Mean values +/- SD are shown. Statistical analysis with student´s t-test revealed no significant differences between treatment and control for each cell line.

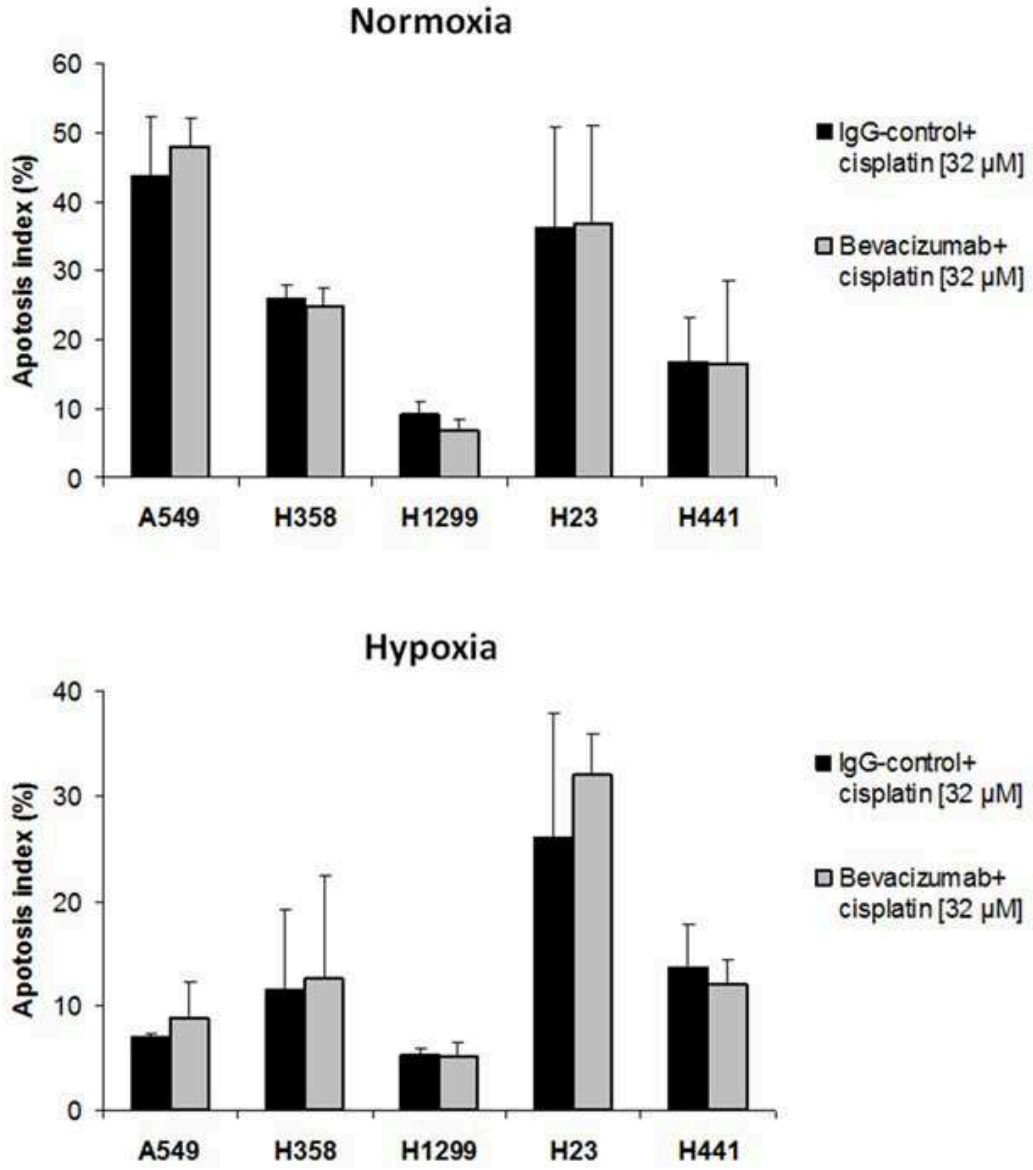


Fig. 24. Effect of bevacizumab on cisplatin induced apoptosis. Normoxic and hypoxic NSCLC cell lines were treated for 3 days with cisplatin [32 µM] and bevacizumab [250 µg] or control. Thereafter, cells were stained with PhiPhiLux® for active caspase-3 and analysed with flow cytometry. Mean values +/- SD are shown. Statistical analysis with student´s t-test revealed no significant differences between treatment and control for each cell line.

5. DISCUSSION

We used two independent methods to investigate proliferation in our cell model and found concordant results throughout the study. We also used two independent methods for assessment of apoptosis. The TUNEL assay detecting DNA single strand breaks and PhiPhiLux® labeling active caspase-3. Both produced consistent results throughout the study. The RNA expression of pro- and anti-apoptotic genes were done in cooperation with Mag.rer.nat. Dr.scient.med. Alexander Deutsch, who performed the qPCR and statistics for this experiment.

In order to answer the questions raised we created a cell model by exposing NSCLC cell lines to hypoxia for a predefined time and compared these cells to controls cultured in normal conditions (normoxia). Optimal time-points and concentrations for the model were assessed via time-course and drug titrations in preliminary experiments. At the beginning we used two NSCLC cell lines widely used in lung cancer studies (Song, Liu et al. 2006, Skvortsova, Skvortsov et al. 2004). Later on, the number of cell lines was extended to six to further reduce the risk of observing only cell line specific phenomena and thereby improve the clinical relevance of the data.

When effects of chronic hypoxia are investigated, both the oxygen level and the maintenance of the same oxygen level throughout the study are very important. It is known that even short periods of re-oxygenation can disturb hypoxic signaling (Toffoli, Michiels 2008). We employed a sophisticated closed incubation system including integrated incubators to perform our experiments in a seamless and stable hypoxic condition.

It is an open question which oxygen level is most suitable to simulate the conditions of NSCLC cells *in situ*. The physiologic range of oxygen concentration in tissues is assumed to be around 5% (Carrera, de Verdier et al. 2010) while 3% might already represent hypoxic conditions (Wang, Zhu et al. 2010). Vaupel (Vaupel 2008) suggested oxygen levels below 1% for induction of adaptational

processes in cancer cells and below 0.1% for selection of hypoxia-resistant cells. Intra-operative pO_2 measurements show that oxygen concentrations around 1% may be more common in lung cancer than lower levels of oxygen (Le, Chen et al. 2006). Therefore we decided to use 1% oxygen in our studies to mimick the in-vivo conditions of NSCLC in patients as good as possible. This decision was supported as Graves et al. (Graves, Vilalta et al. 2010) recently stated that average oxygen levels of lung tumors are higher than those of other solid tumors. In primary NSCLC tumors they measured a median pO_2 of 13.5 mmHg, which corresponds to an oxygen concentration slightly below 2%. Nevertheless it cannot be excluded that both conditions, extreme and moderate hypoxia, occur *in vivo* as it's likely that there is a gradient of oxygen tension towards the center of a solid tumor, which may have no lower limit.

In our opinion it is of importance if resistance to chemotherapy under hypoxic conditions occurs due to selection or due to adaptation. If selection is most predominant *in vivo* it would imply that this process is not reversible. The pressure of extreme hypoxia (and likely also nutrient deprivation) leads to apoptosis and only the "strongest", mostly multi-resistant cells survive. In this case new strong drugs would be needed to kill these cells. On the other hand, if the mechanism is a process of adaptation and we manage to reveal the mechanisms of this adaptation, one could maybe reverse this process and avoid the resistance.

Hypoxia-induced chemoresistance has been studied intensively in the past years. Graeber et al. (Graeber, Osmanian et al. 1996) applied 0.02% O_2 to rat fibroblasts with and without p53 deficiency and saw that the majority of p53 wild-type cells went into apoptosis whereas the fibroblasts with loss of p53 did not. Furthermore they found that they could select the p53 deficient cells out of a co-culture with p53 wild-type cells by applying repeated circles of normoxia and hypoxia. Weinmann et al. (Weinmann, Jendrossek et al. 2004) could show that hypoxia driven selection even occurs in genetically unmodified NCI-H460 lung cancer cells. The selected cells were also shown to be more resistant against radiation and etoposide. However, the mechanism was found to be independent of p53 status. Kim et al.

(Kim, Tsai et al. 1997) found a hypoxia-induced selection process in human cervical epithelial cells.

In contrast, Alvarez-Tejado et al. (Alvarez-Tejado, Naranjo-Suarez et al. 2001) did not find apoptosis induction by hypoxia (1% O_2) *per se* but nevertheless rat pheochromocytoma PC12 cells kept in hypoxia grew resistant to serum-deprivation and fluorouracil (5-FU) involving a PI3K/AKT depending pathway. Similar results were obtained in rhabdomyosarcoma and Ewing sarcoma cell models involving HIF-1α (Kilic, Kasperczyk et al. 2007). The group around Carine Michiels emphasised the role of p53, c-jun and HIF-1α in adaptation to hypoxia and consequent resistance to apoptosis in HepG2 cells (Sermeus, Cosse et al. 2008, Cosse, Ronvaux et al. 2009). Moreover resistance to taxol and cisplatin in hypoxic ovarian cancer cells was shown to be dependent on STAT3 (Selvendiran, Bratasz et al. 2009). For chemoresistance induced by moderate hypoxia in A549 NSCLC cells, contradictory results have been published (Lee, Lee et al. 2006, Cosse, Sermeus et al. 2007). In this study we show that in conditions of 1% O_2 the NSCLC cell lines A549 and NCI-H358 do not undergo apoptosis or necrosis for at least 72 h (Fig. 8 and 9). Furthermore, these conditions result in a marked resistance against cisplatin-induced apoptosis (Fig. 10-13). For the first time in this study we could show that this resistance to cisplatin is reversed after re-oxygenation in NSCLC cells in a time-dependent manner (Fig. 14). This is in agreement with previous data on human melanoma cell lines treated with doxorubicin and methotrexate (Sanna, Rofstad 1994) and suggests that our experimental conditions induced adaptation to hypoxia and that selection played no major role.

Recently it has been shown that proliferation of cancer cells is inhibited by hypoxia and that this inhibition might lead to reduced efficacy of cell cycle dependent drugs (Wen, Ding et al. 2010). In our study we inhibited the growth rate of normoxic cells by serum starvation, mimicking the proliferation inhibition by 1% oxygen. The hypothesis was that hypoxia might cause a growth rate independent cisplatin resistance. Indeed, we found that the ability of cisplatin to induce apoptosis in NSCLC cells is largely independent of the growth rate (Fig. 15). The same conclusion was drawn from the comparison of A549 and NCI-H358 cells (Fig. 8-

13). The adenocarcinoma cell line A549 is rapidly growing (doubling time: 22 h) while NCI-H358 cells have a low proliferation rate (doubling time: 38 h). We found that growth rate *per se* had no effect on hypoxia-induced cisplatin resistance nor on the cisplatin-induced apoptosis rate in normoxia. This is an unexpected finding if we consider that cisplatin is a DNA-damaging agent. We used therapeutically relevant cisplatin concentrations (Verschraagen, Boven et al. 2003). At these concentrations, cisplatin might not only damage the DNA, but also mitochondria (Cullen, Yang et al. 2007). Moreover, it has been shown that cisplatin induces apoptosis even in enucleated cells (Fuertes, Castilla et al. 2003). Berndtsson et al. showed that cisplatin at concentrations above 10 µM induces apoptosis that seems to be independent from DNA-damage but maybe due to reactive oxygen species production and protein adduct formation (Berndtsson, Hagg et al. 2007).

There are several explanations for hypoxia-induced cisplatin resistance. One of the key players for hypoxia-mediated effects is HIF-1α (Vaupel, Mayer 2007, Semenza 2010a, Semenza 2010b, Vaupel 2008). Several studies show that HIF-1α is involved in hypoxia-induced chemoresistance and that knocking down HIF-1α can abrogate the resistance (Hussein, Estlin et al. 2006, Kilic, Kasperczyk et al. 2007, Wen, Ding et al. 2010, Zhang, Zhang et al. 2004). Consistent with these data we found an up-regulation of HIF-1α in our cell lines under hypoxic conditions (Fig. 17A).

The exact downstream mechanisms how HIF-1α facilitates resistance to chemotherapy in hypoxia are unknown.

As indicated in the introduction the decision if a cell goes into apoptosis or not is dependent on the balance between pro- and anti-apoptotic proteins of the BCL-2 family. The hypothesis that hypoxia-induced apoptosis resistance is due to a decrease of pro- or an increase of anti-apoptotic factors is nearby. Several studies claimed the involvement of pro-and anti-apoptotic proteins in adaptation mediated as well as in selection mediated hypoxia-induced chemoresistance, both HIF-1α dependent and independent. (Zhang, Zhang et al. 2004, Dong, Venkatachalam et al. 2001, Erler, Cawthorne et al. 2004, Sermeus, Cosse et al. 2008, Kim, Ahn et al. 2004, Bruick 2000, Sowter, Ratcliffe et al. 2001).

Our data suggest that down-regulation of the pro-apoptotic protein BAX plays a role in hypoxia-induced chemoresistance. This is in line with Kim et al. (Kim, Park et al. 2004) showing that inhibition of BAX function is a target mechanism through which hypoxia inhibits TRAIL-induced apoptosis. Furthermore Sasabe et al. (Sasabe, Tatemoto et al. 2005) have shown that overexpression of HIF-1α induced downregulation of BAX in human oral squamous cell carcinoma cells.

Cancer cells secrete VEGF to induce angiogenesis in their stroma to facilitate nutrient and oxygen supply for the fast growing cancer cell mass. Tumor growth without vascularisation is slow (Folkman 1990). Bevacizumab (Avastin®) was shown to inhibit neo-angiogenesis, force regression of immature vessels and normalize existing vessels leading to tumor growth inhibition and enhance the accessibility of the tumor for chemotherapeutics (Willett, Boucher et al. 2004, Mancuso, Davis et al. 2006, Jain 2005). Avastin® is already clinically approved for use in mostly late stage tumors of the lung (NSCLC, no squamos cell), colon, breast and kidney.
Already in the year 1999 Decaussin et al. (Decaussin, Sartelet et al. 1999) investigated the prognostic importance of the expression of VEGF and its receptors in NSCLC. In their study no prognostic significance of these factors was found but they already suggested that VEGF might act as direct autocrine growth factor for tumor cells. Indeed two years later Masood et al. (Masood, Cai et al. 2001) proofed this to be true *in vitro* for human melanoma, ovarian carcinoma, pancreatic carcinoma and Kaposi sarcoma cell lines that express VEGF receptors and produce VEGF using VEGF siRNA and VEGF receptor 2 neutralizing antibodies. Again with VEGF siRNA but VEGF neutralizing antibodies Castro-Riviera et al. (Castro-Rivera, Ran et al. 2004) showed growth inhibition in lung cancer and breast cancer cell lines. Finally Liu B et al. (Liu, Peng et al. 2009) downregulated VEGF-A by restoration of MiR-126 and found growth inhibition of lung cancer cell lines *in vitro* and *in vivo*.

However, in our study we could not inhibit proliferation of NSCLC cell lines using bevacizumab in a concentration higher than needed for scavenging the VEGF

secreted by the NSCLC cell lines in 3 days (Fig. 18 and 21). Even cell lines (H1299) that were known from the literature to respond with reduced proliferation to abrogating the VEGF loop, did not show the expected result.

We have shown in this study with two methods that the cell lines we used express VEGF receptor 1 (Fig. 19 and 20). After the negative proliferation results with bevacizumab the question if the receptors are also functional was nearby. Therefore the cell lines were stimulated with human VEGF and analyzed with thymidin-uptake. Only one out of the six cell lines responded significantly and dose-dependently to this stimulation (Fig. 22). This could explain the negative results obtained. However, also in the cell line reacting to VEGF stimulation, proliferation could not be inhibited with bevacizumab.

Besides autocrine proliferative effects of a putative VEGF loop, also pro-survival and anti-apoptotic mechanisms have been proposed. Bachelder et al. (Bachelder, Crago et al. 2001) published that VEGF expression is essential for the survival of metastatic breast cancer cell lines *in vitro* and that suppression of VEGF expression induced apoptosis in these cells. It was shown that blocking VEGF/VEGFR1 signaling induces apoptosis in colon cancer cells undergoing epithelial-mesenchymal transition in a spheroid model (Bates, Goldsmith et al. 2003). Using VEGF siRNA or VEGFR tyrosine-kinase inhibitors bladder cancer and metastatic melanoma cancer cell lines could be sensitized to cisplatin treatment (Sini, Samarzija et al. 2008, Krause, Forster et al. 2005).

This also raised our interest to investigate if blocking the autocrine VEGF loop would induce apoptosis in NSCLC cell lines. Instead of going for siRNA or tyrosine-kinase inhibitors we decided that an already clinically approved drug would be of higher interest, namely bevacizumab. Incubating NSCLC cells with bevacizumab for 3 days did not induce apoptosis in our experimental setup (Fig. 23). Also co-treatment with cisplatin did not reveal any sensitizing effects of bevacizumab in our study (Fig. 24). The bevacizumab concentration we used was sensible and clinically relevant in our eyes. Avastin® is used in the clinics in a concentration of 7,5 mg/kg bodyweight. A patient with 70 kg has roughly 5 liters of blood volume and hence a full dose of 525 mg bevacizumab which would lead to a “serum-level” of about 100 µg/ml, of course not considering a further dilution in the periphery. For

an adipose patient this could go up to maybe 200 µg/ml. Yoeruek et al. (Yoeruek, Tatar et al. 2010) used bevacizumab in a concentration of 5 mg/ml on human corneal endothelial cells *in vitro* and did also see no apoptosis induction upon this treatment. Nevertheless our findings are in contrast to the other studies discussed above.

In two studies on breast and colorectal cancer cell lines a new mechanism of VEGF survival signaling was proposed, namely “intracrine signaling”. Lee et al. (Lee, Seng et al. 2007) found in breast cancer cell lines an increased apoptosis rate when VEGF expression was downregulated with antisense VEGF cDNA or with siVEGF. Also siRNA against VEGFR1 lead to reduced survival of breast cancer cells, whereas targeting VEGFR2 or neuropilin 1 had no effect. Interestingly they could not induce the same effects with stimulating or inhibiting extracellular/autocrine signaling with placenta growth factor or VEGFR1 antibodies. Assessing the localization of VEGFR1 they found it to be intracellular. Samuel et al. (Samuel, Fan et al. 2011) obtained similar results in colorectal cancer cell lines: Downregulation of VEGF expression lead to growth inhibition, apoptosis augmentation and sensitization to 5-FU. However also they could not induce effects using bevacizumab (250 µg/ml) or even intracellular tyrosine-kinase inhibitors. Their explanation is that the signaling could be intracellular involving neuropillin.

Hence, exploring a possible intracrine loop of VEGF in NSCLC lines and the importance of neuropillin receptors should be future goals of our research.

In this study we show that chronic moderate hypoxia induces resistance to cisplatin in A549 and NCI-H358 NSCLC cells without involvement of selection pressure and is associated with down-regulation of the pro-apoptotic protein BAX. These effects are fully reversible after 24-48h of re-oxygenation. Hypoxia reduces the growth rate of A549 cells and, insignificantly, NCI-H358 cells and the growth rate of A549 cells was significantly higher than of NCI-H358 cells. Despite this hypoxia-induced cisplatin resistance was independent of growth rate. Serum starvation mimicked the effect of hypoxia on cell proliferation, however, starvation did not inhibit

cisplatin-induced apoptosis. This indicates that hypoxia-induced cisplatin-resistance is independent of proliferation rate and must involve a reversible molecular mechanism in these cancer cells.

Furthermore, we show in this study that NSCLC cell lines produce and secrete VEGF. NSCLC cell lines express VEGFR1 whereas VEGFR2 is only weakly expressed. In this study the clinically approved drug bevacizumab (Avastin®) could neither inhibit proliferation of NSCLC cells nor induce apoptosis *in vitro*. Moreover, bevacizumab could not enhance cisplatin-induced apoptosis in cell culture experiments.

6. CONCLUSIONS

This study shows that hypoxia-induced cisplatin resistance is specifically induced by a reversible molecular mechanism involving HIF-1α and BAX. This could give rise to new therapeutic strategies in the treatment of NSCLC.

The data obtained in this study questions if the effects of bevacizumab, as putative inhibitor of an autocrine VEGF signaling loop, are more pro-apoptotic than anti-angiogenic although a possible intracrine signaling was not investigated.

7. PUBLICATIONS

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Wohlkoenig C, Leithner K, Deutsch A, Hrzenjak A, Olschewski A, Olschewski H. Hypoxia-induced cisplatin resistance is reversible and growth rate independent in lung cancer cells. Cancer Lett. 2011;308:134-43.

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