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DISSERTATION

The role and influence of pigment epithelium-derived factor (PEDF) on peripheral nerve tumor, brain trauma and stroke

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List of Abbreviations

aCSF = Artificial Cerobrospinal Fluid
ANOVA = Analysis of variance
BrdU = Bromo-deoxy-Uridine
CCI = Controlled Cortical Impact Injury
CD31 = Cluster of Differentiation-31
cDNA = Complementary Deoxyribonucleic Acid
DG = Dentate Gyrus
ED1 = Ectodermal Dysplasia-1
MCAO = Middle Cerebral Artery Occlusion
MMP9 = Matrix Metalloproteinase-9
MPNST = Malignant Peripheral Nerve Sheath Tumor
MTT = 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
mRNA = Messenger Ribonucleic Acid
NF1 = Neurofibromatosis Type-1
PBS = Phosphate Buffered Saline
PEDF = Pigment Epithelium-Derived Factor
PFA = Paraformaldehyde
RT-PCR = Real Time-Polymerase Chain Reaction
SD = Standard Deviation
SEM = Standard Error of Mean
SPSS = Statistical Package for the Social Sciences
SVZ = Subventricular Zone
TBI = Traumatic Brain Injury
TUNEL = Terminal deoxynucleotidyl transferase dUTP Nick End Labelling
VEGF = Vascular Endothelial Growth Factor

ZUSAMMENFASSUNG

Der Pigment epithelium-derived factor (PEDF) ist ein neurotropher Faktor, der Gehirnzellen schützt, die Bildung neuer Gefäßstrukturen und die Entstehung von Tumoren verhindert sowie entzündungshemmend wirkt. Unser Ziel war es, die Auswirkungen von PEDF auf die Gefäßbildung und das Tumorwachstum bei malignen peripheren Nervenscheidentumor (MPNST) sowie auf das Läsionsvolumen, Zelltod, Zellteilung und Verhalten bei Hirnschädigungen (Schädelhirntrauma und Schlaganfall) zu zeigen. Schädelhirntrauma wurde experimentell durch kontrollierte kortikale Kontusion (CCI) in der Ratte herbeigeführt und der Schlaganfall wurde mittels Mausmodell des Verschluss der mittleren Hirnarterie (MCAO) untersucht.

In der ersten Studie zeigten wir erstmals, dass sich kultivierte S462 MPNST Zellen, die 48 Stunden lang mit PEDF behandelt wurden, weniger stark teilten und stärker abstarben. Nach Transplantation der S462 MPNST Zellen in athymische Naktmäuse verringerte PEDF außerdem die Belastung durch den MPNST Tumor. Dies war auf eine Verringerung der Bildung neuer Gefäßstrukturen zurückzuführen.

In der zweiten Studien stellten wir eine signifikant erhöhte mRNA-Expression von PEDF nach CCI in Rattengehirnen fest. Infusion von PEDF führte zu einer nichtsignifikanten Verringerung des Kontusionsvolumens nach CCI, während sich die Zahl der toten Zellen, aktivierten Microglia und BrdU-positiven Zellen um die Läsion signifikant verringerte. Im Gegensatz dazu war die Zellteilung in der ipsilateralen Suventrikularzone signifikant erhöht.

In der dritten Studie führte die MCAO zu einem striatalen Schlaganfall in der Maus. Wir stellten keine Reduktion des Infarktvolumens und des Zelltods nach experimentellem Schlaganfall zwischen Tieren, die eine Infusion von PEDF oder Zerebrospinalflüssigkeit erhielten, fest. Ebenso gab es keine Unterschiede im Verhalten oder der Zellteilung zwischen den beiden Gruppen.

Während PEDF einen positiven Effekt nach Schädelhirntrauma in der Ratte zeigte, konnten wir diesen Effekt nach striatalem Schlaganfall in der Maus nicht bestätigen. Jedoch können wir nicht ausschließen, dass PEDF unter anderen Bedingungen auch beim Schlaganfall wirksam sein kann. Weiterführende Studien sind notwendig, um die Auswirkungen der PEDF-Behandlung bei moderatem bis schwerwiegendem Schlaganfall aufzuklären. Aufgrund seines inhibitorischen Effekts auf das Wachstum von humanen MPNSTs, scheint PEDF ein vielversprechender Wirkstoff zur Therapie von MPNST zu sein.

ABSTRACT

Pigment epithelium-derived factor (PEDF) is a neurotrophic factor with neuroprotective, antiangiogenic, anti-inflammatory, and anti-tumorigenic effects. We aimed to show the effects of PEDF on angiogenesis and tumor growth of malignant peripheral nerve sheath tumor (MPNST) as well as on lesion volume, cell death, cell proliferation, and behavioral outcome after brain injuries (traumatic brain injury and stroke). We used the controlled cortical impact injury (CCI) rat model to study traumatic brain injury and the middle cerebral artery occlusion (MCAO) mouse model for focal cerebral ischemia.

In the first study, we demonstrated for the first time that PEDF inhibited proliferation and augmented cell death in S462 MPNST cells after 48 h of treatment in culture. Following transplantation of S462 MPNST cells in athymic nude mice, PEDF reduced MPNST tumor burden mainly due to inhibition of angiogenesis.

In the second study, we detected a significant increase of PEDF mRNA levels in post-CCI rat brains. *In vivo*, PEDF infusion showed no significant decrease in the contusion volume, whereas the number of dead cells, activated microglia, and BrdU-positive cells around the lesion was significantly decreased. In contrast, PEDF infusion significantly increased cell proliferation in the ipsilateral subventricular zone.

In the third study, our model produced an ischemic injury confined solely to striatal damage in mice. We detected no reduction in infarct size and cell death in PEDF- vs. cerebrospinal fluid-infused MCAO mice. Behavioral outcome and cell proliferation did not differ between the groups.

Whereas PEDF showed a specific positive effect after traumatic brain injury in rats, we were not able to observe the same effect after striatal ischemia in the MCAO mouse model. However, we cannot exclude that PEDF might work under different conditions in stroke. Therefore, further studies will elucidate the effect of PEDF treatment on cell proliferation and outcome in moderate to severe ischemic injury in the brain. Due to its inhibitory effects on the growth of human MPNST, PEDF seems to be promising for future therapeutic purposes against MPNST.

INTRODUCTION

Pigment epithelium-derived factor (PEDF), a non-inhibitory member of the serine protease inhibitor (serpin) family, is a neurotrophic, neuroprotective, anti-tumorigenic, and antiangiogenic factor[1-3]. PEDF was originally purified from human retinal pigment epithelial cell-conditioned media[4]. Although the spatiotemporal expression of PEDF receptors remains unclear, most mammalian tissues produce PEDF, including brain and particularly striatum[5], cerebellum[6], hippocampus and hypothalamus[7] as well as cerebral cortex[8, 9].

PEDF is a promising therapeutic agent in neurodegeneration, angiogenesis, and inflammation-related pathologies[3, 10, 11]. It is aberrantly expressed in ocular disorders such as ischemia-induced retinal neovascularization and diabetic retinopathy[12, 13]. Down-regulation of PEDF was recently shown in a range of cancers[14, 15].

PEDF can function as a tumor suppressor protein in several types of cancer, including neuronal tumors[16, 17]. A recent study in an osteosarcoma model showed that PEDF treatment induces apoptosis, inhibits cell cycle progression *in vitro* and reduces tumor burden *in vivo*[18]. In a trial with bladder cancer patients, PEDF expression significantly decreased in the urothelium, whereas the expression of pro-angiogenic factors including vascular endothelial growth factor (VEGF) and matrix metalloproteinase-9 (MMP-9) increased. Tumorigenesis in these cancer patients can be caused by downregulation of PEDF expression, which leads to the loss of differentiation in urothelial cells of the bladder, uncontrolled proliferation, and induction of angiogenesis with increased VEGF and MMP-9 expression[15].

Recent reviews have also summarized the antitumor effects of PEDF with respect to anti-angiogenesis, apoptosis, and differentiation activities[19, 20]. Until now, effect of PEDF on MPNST has not been evaluated yet. In this thesis I investigated the effects of PEDF on angiogenesis and tumor growth in MPNST.

In vitro and *in vivo,* PEDF is neuroprotective after a variety of neuronal injuries[6, 21]. However, its effect on traumatic brain injury (TBI) has not been evaluated yet. Among other effects, PEDF protects neuronal cells by preventing cell death and inflammation[22, 23]. Early after TBI, activation of microglial cells occurs[24]. In mice and rat models of TBI, the number of activated microglia in both ipsi- and contralateral hemispheres significantly increases compared to control mice[25, 26]. In rat microglia-astrocyte culture,

PEDF treatment induces activation of microglia but at the same time inhibits proliferation of both microglial cells and astrocytes[27].

Moreover, PEDF promotes self-renewal of neuronal stem cells in culture. *In vivo* infusion of PEDF in mice induces cell proliferation by increasing the number of B-cells residing in the subventricular zone (SVZ), which represent the multipotent neuronal stem cells[28]. It is speculated that endogenous PEDF, secreted from ependymal cells in SVZ and endothelial cells, promotes neurogenesis in the brain[28]. In this thesis, I investigated the neuroprotective effects of PEDF in rats after TBI.

In the third study, we aimed to show how PEDF influences cell proliferation and infarct size in mice after experimental stroke. We used the middle cerebral artery occlusion (MCAO) model, the most commonly used model of ischemic stroke. In permanent MCAO in the rat, intrinsic PEDF protein and mRNA levels are downregulated in post-stroke brains[29]. In another study, PEDF gene delivery significantly decreases infarct size and edema, attenuates cell death, and reduces inflammation after MCAO[6]. One of the underlying mechanisms of PEDF action is its regulatory effect on cellular proliferation. PEDF promotes self-renewal of adult neuronal stem cells in the brain[28]. Furthermore it increases metabolic activity in microglia while blocking proliferation[27].

In this thesis, I aimed to investigate; 1) the anti-tumorigenic effects of PEDF on angiogenesis and tumor growth of MPNST; 2) the neuroprotective effects of PEDF on, lesion volume, cell death, microglia activation, and cell proliferation after TBI in rats; and 3) on infarct size, behavioral outcome, cell proliferation and cell death after MCAO in mice. Overall, with these 3 studies, we investigated the effect of PEDF as a multifunctional neuroprotective factor in MPNST, brain trauma and stroke in order to enlighten its promising therapeutic role in similar human pathologies.

MATERIALS AND METHODS

All materials and methods used are described in the selected publications in detail. In this section, only the main materials and methods related to the results, models and experimental aims are summarized.

Cell lines, animals, experimental design and ethical statement

We performed all animal experiments in consent with national and international guidelines for the care and use of laboratory animals (Landesamt für Gesundheit und

Soziales, Berlin, Germany). We treated the animals with respect to the German Animal Protection Act by European Council Directive (86/609/EEC) concerning the protection of experimental animals and by the Animal Experimentation Commission of the Medical Faculty of the University of Regensburg and the Government of Oberpfalz (Subject 54, Veterinary).

In publication-1, eight weeks old male athymic mice were used for the xenograft model experiment. For the *in vitro* and xenograft experiments, we used human malignant S462 MPNST cell lines. We cultured S462 MPNST cells were cultured in the presence or absence of recombinant PEDF. After 3 days, cell viability and cell death were measured. We subcutaneously injected S462 MPNST cells into the right hind flanks of athymic mice. Mice received phosphate-buffered saline (PBS, as control) or PEDF protein injected directly into the tumors. We measured tumor size using calipers. Seven days after injections, tumors were resected and sliced for immunohistochemical analysis of cell death and microvascular density.

In publication-2, two-month-old adult male Fischer rats (F-344, 240-270 g body weight) were separated into two groups: sham operated rats, subjected to micro-drill skull trepanation without damaging the dura mater; and traumatized rats, subjected to unilateral controlled cortical impact injury (CCI) on the cerebral cortex of the right hemisphere. After the operations, rats were prepared either for real time PCR (RT-PCR) to assess mRNA expression levels of PEDF, at 4h, 1, 4, and 7 days after CCI; or for osmotic pump implantation to receive 5 or 10 µg/ml of PEDF or aCSF (vehicle) into the contralateral ventricle (left hemisphere) or no pump as well as intraperitoneal injections of Bromo-deoxy-Uridine (BrdU) for a period of 8 days, including operation day. Detection of BrdU (for proliferating cells), ED1 antigen (for activated microglia) and dead cells (TUNEL assay) were performed on the latter group of animals.

In publication-3, eight- to eleven-week-old male C57BL6/N mice were used to induce transient MCAO. Twelve mice underwent osmotic pump implantation two days prior to MCAO. Starting at the day of the MCAO, mice received daily intraperitoneal injections of BrdU for 7 days. 24 hrs after reperfusion, we performed T2-weighted magnetic resonance imaging (MRI). On day 5 after MCAO, we removed the osmotic pumps. Animals underwent behavioral testing from day 7 to day 21. We sacrificed the mice and extracted their brains for tissue analysis on day 21 after MCAO. The behavioral tests were based on own previous results of our group and the literature[30, 31]. We used Open Field test on day 7 to evaluate exploratory locomotion and anxiety. Pole test on day 9

and rotarod on day 10 were chosen to assess motor function. Using Morris Water Maze (MWM), we investigated spatial learning and memory. We performed training for MWM for seven days prior to probe trial on day 21.

Controlled cortical impact injury

In publication-2, we used a standard CCI model as previously described[32], to investigate PEDF expression and the effect of PEDF infusion in posttraumatic rat brains. All animals were anesthetized by intraperitoneal injection of an anesthetic cocktail. For surgery, the animals were positioned in a stereotactic frame. Then, we performed a 6 x 3 mm craniotomy over the right hemisphere[33]. We used a pneumatic and computer driven piston to perform a moderate contusion. The pneumatic bolt was positioned with an angle of 45° and perpendicular to the surface of the cortex. We adjusted the bolt's penetration depth to 2 mm below dura level (diameter of convex bolt, 7 mm; velocity, 6.5 m/s; contact time, 150 ms and pressure 3.5 bar). The dura matter remained intact after the impact.

Osmotic pump implantation

In publications 2 and 3, we used osmotic mini pumps for intracerebroventricular delivery of PEDF or aCSF.

In publication-2, the pump implantation was performed immediately following cortical trauma to reduce stress of animals by decreasing number of operations under general anesthesia. The infusion of cannula was positioned by using stereotactic system and guided by bregma and lambda as reference points (+1.4 lateral, -0.8 anterioposterior, -5.0 dorsoventrally). Rats in PEDF5 and PEDF10 groups were daily infused with 0.12 ± 0.018 and 0.24 ± 0.036 µg PEDF protein respectively. We explanted all pumps at day 7 post operation.

In publication-3, we implanted the pumps two days prior to MCAO. The cannula was located 0.2 mm to the right from Bregma corresponding to the location of the right ventricle. The animals received a total amount of 84±16.8 µl of PEDF (20 µg/ml in CSF) or CSF. On day 7 after MCAO, we removed all osmotic pumps.

BrdU injection

Rats and mice used for immunohistochemistry received daily intraperitoneal BrdU injections to assess cell proliferation (50 mg per kg body weight, diluted in 0.9% NaCl solution).

Quantitative RT-PCR

In publication-2, we sacrificed rats used for expression studies at 4 h, 1, 4, and 7 days after CCI. For RNA extraction, a well defined portion of contusion was snap frozen in liquid nitrogen. We extracted RNA from rat brains according to the manufacturer's protocol. RNA was reversed transcribed to complementary DNAs (cDNAs) which we used for RT-PCR in order to measure expression levels of PEDF in a semi-quantitative manner. We normalized gene expression to housekeeper rat ß-actin gene.

Cell proliferation/viability assay

In publication-1, S462 MPNST cells (10^4 cells/well) were seeded in 96-well plates in the presence or absence of recombinant PEDF (10, 20, 50, 100 and 200 ng/ml; triplicates per condition). After 3 days, we added MTT solution (5 mg/ml) to each well and the plates were further incubated for 3 h. We measured absorbance with a microplate reader. We calculated the value of the treated cells as a percentage of the untreated control.

In vivo testing of anti-tumoral effects: Xenograft model

In publication-1, we injected S462 MPNST cells (5×10^6) subcutaneously into the right hind flanks of athymic mice. When the tumors reached approximately 90 mm^3, mice randomly received PBS as control (n=5) or PEDF protein (n=8). We monitored the tumors daily for the first week and then every two days. Tumor size was measured with callipers.

Middle Cerebral Artery Occlusion

In publication-3, we induced MCAO by inserting a silicone rubber-coated 190 µm thick monofilament via the internal carotid artery as described previously[34]. Mice were anesthetized with an anesthetic cocktail. After 60 min of ischemia, we re-anesthetized the animals and removed the filament to permit reperfusion. During surgery and ischemia, body temperature was kept between 37.0 and 37.5°C.

Magnetic Resonance Imaging

In publication-3, we scanned mice 24 hrs after MCAO in a 7 Tesla Pharmascan 70/16 equipped with a 16 cm horizontal bore magnet and a 9 mm (inner diameter) shielded gradient. We carried out data acquisition and image processing with the Bruker software. For imaging the mouse brain, we used a T2-weighted 2D turbo spin-echo sequence (TR/TE: 4200/36 ms, rare factor 8, 4 averages). Twenty 0.5 mm-hick axial slices over the brain from olfactory bulb to cerebellum were imaged with a field of view of 2.56 x 2.56 cm and a matrix size of 256 x 256, resulting in a nominal voxel size of 98 µm x 98 µm. The acquisition of the T2-weighted images lasted 6 min 43 s.

Behavioral Tests

1. Open Field Test: It consists of an unfamiliar open field (50x50 cm) surrounded by high walls. On day 7 after MCAO, mice were given one trial to explore the field freely for 10 min. We monitored locomotor activity using a video tracking system and recorded the total distance each mouse traveled and time spent in the open center of 35x35 cm.

2. Pole Test: It was performed as previously described[35], with minor modifications. The test apparatus is a vertical steel pole covered with tape to create a rough surface. On day 9 after MCAO, we placed the mice head upward on the top of the pole. We recorded the time the mouse took to turn completely head downwards and the total time it took to descend down and reach the floor with its front paws. If the animal was unable to turn completely, we attributed the time to reach the floor to *t* turn, too.

3. Rotarod: We performed Rotarod as previously described[36]. On day 10 after MCAO, we placed the mice on an accelerating rotating rod (from 4 to 40 rpm over 300 s) and their latency to fall was recorded.

4. Morris Water Maze Test: It was performed as described with minor modifications[37]. We filled a circular swimming pool (120 cm Ø, 60-cm-high, 32 cm depth) with 20°C opaque water. We fixed visible cues on the pool walls. A Plexiglas platform (11 cm Ø) was submerged 1 cm below the water level. 2-phase experiment consists of a place task (learning period) with three trials per day for seven consecutive days (day 14-20 after MCAO) and a probe trial (spatial probe) on the eighth day (day 21 after MCAO). For the learning period, we placed the platform in one of the quadrants and released the mice into the water from one of the other quadrants to search for the platform. After reaching the platform, we allowed the animals to remain there for 30 s. We recorded escape latencies to find the platform, total distance travelled and swim speed. In the

probe trial, we let mice to swim freely for 90 s in the absence of the platform. We measured the time spent in each quadrant and crosses through the location of the former platform.

Histology and immunohistochemistry

Animals were perfused with saline (0.9% NaCl) followed by 4% PFA under deep anesthesia. Thereafter, we incubated the brains in 4% PFA overnight and in 30% sucrose solution at 4°C until brains started to sink. Brains were deep frozen by means of methylbutane on dry-ice and then kept at -80°C until cutting. We covered the brains with a cryo-protectant.

In publication-1, seven days after injection of PBS (n=5) or PEDF (n=6) into the MPNST-tumor tissues, tumors were resected and fixed overnight in 10% formalin, washed in PBS and embedded in paraffin. We deparaffinized 4µm-thick paraffin sections in xylol and a descending series of ethanol for further immunohistochemical analysis. We used TUNEL assay to mark dead cells and anti-CD31 antibody to count microvessels.

In publication-2, we collected 30 µm thick coronal rat brain slices as floating-sections. From each brain, we selected 12 slices at a distance of 0.5 mm throughout lesion area (between Bregma -0.40 and -6.3 mm) for the histology[33]. The floating sections (3-6 depending on size) were transferred into petri dishes containing PBS and then transferred onto the Superfrost slides. We used cresyl violet staining to assess tissue structure and to measure lesion volume. We calculated contusion volume by multiplying contusion areas with the distance between histological sections (500 µm). TUNEL staining was used to mark dead cells. We used anti-ED-1 antibody to detect microglial cells and anti-BrdU antibody for proliferating cells.

In publication-3, we cut 20 µm thick coronal slices. To detect proliferating cells, we used anti-BrdU antibody and TUNEL to detect dead cells. For identification of proliferating cells, we used anti-BrdU antibody with anti-GFAP for astrocytes, anti-NeuN for neurons and anti-Iba1 for microglial cells as double stainings.

Microscopy and image analysis

For all microscopic counting, two tissue slices per animals were used according to a previously described counting method[38]. All measurements were made by an investigator blinded to the injury status and treatment regimens of the animals.

In publication-1, we assessed the number of CD31-positive microvessels and TUNEL-positive cells with a microscope. We calculated the area of CD31-positive staining by using threshold color measurement with Cell P software.

In publication-2, we counted ED1-postive microglia, BrdU-positive cells, and TUNEL-positive cells in the lesion area, SVZ, and dentate gyrus (DG) of the hippocampus using stereoinvestigator on a microscope. We selected 12 slices at a distance of 0.5 mm throughout the lesion area for histology[33]. All cells in the area SVZ and DG and 10 fields of view of the lesion site per slice were counted. Cell numbers were calculated as cells/mm².

In publication-3, we counted BrdU-positive proliferating cells and TUNEL-positive cells with a microscope. For cortex and striatum, 4 fields of view per slice and for SVZ and DG the whole area were counted. For TUNEL, we subtracted the number of cells in contralateral striatum from ipsilateral striatum and calculated the mean for each animal. For double stainings, we counted the cells in the whole striatum on one slice per animal.

Statistical Analysis

We tested normality using Kolmogorov-Smirnov test and variance homogeneity using Levené test. When data were normally distributed, variance homogeneity was met and more than two groups were analyzed, one-way independent ANOVA with Tukey-HSD post hoc was performed. When two independent groups were investigated, student's t-test was performed. In case of comparing two groups with repeated measures, ANOVA was performed with Box's M test for equality of covariance matrices and Levené test for variance homogeneity. When two groups were analyzed where data was normally distributed, but variances were not homogenous, Mann-Whitney U test was performed. Data are expressed as mean ± standard deviation (SD) or as mean ± standard error of mean (SEM). For the mRNA data in publication-2, we used medians because variance homogeneity was not met and Mann-Whitney U test was performed.

For all studies, we considered a value of $p<0.05$ statistically significant. We performed all statistical analyses with SPSS v.19.0.

RESULTS

The results of the studies are described in detail in the publications listed under "selected publications". In this section only the main findings are summarized and briefly discussed.

Publication-1: "Effects of pigment epithelium derived factor (PEDF) on malignant peripheral nerve sheath tumors (MPNSTs)"

In vitro treatment of MPNST S462 cells with PEDF significantly reduced cell viability (Fig. 1). We observed a significant reduction in the total MPNST cell population following treatment with PEDF at a concentration of 50 ng/ml, with a maximal reduction in cell viability at 100 ng/ml.

Flow cytometric analysis of the cell death marker Annexin V showed that increasing concentrations of PEDF induced cell death in the MPNST cell line (Fig. 2). *In vitro*, we also detected a significant increase in TUNEL-positive cells, confirming the increase in dead cells after PEDF treatment.

MPNST xenografted tumors exhibited typical histological characteristics of MPNST, i.e. tumor cells were spindle shaped, highly proliferative and S100-positive[39]. In addition, hematoxylin-eosin staining revealed that in PEDF-treated tumors, hyaline formations were present in a more disorganized arrangement, as compared to the PBS-treated control tissue (Fig. 3). One week after treatment, we detected a significant reduction in the average volume of PEDF- compared with PBS-treated tumors.

In vivo, PEDF treatment significantly increased the number of TUNEL-positive cells within the MPNST tumor tissue (both tumor and endothelial cells) as compared to PBS treatment (Fig. 4).

To assess whether PEDF had an effect on blood vessel formation, we stained sections with CD31, an endothelial cell marker. We observed small and frail appearing vessels within PEDF-treated tumor tissue, in contrast to the more typical tumorigenic vasculature within the PBS-treated tumors (Fig. 5). PEDF significantly reduced the total area of CD31-positive vessels per mm^2 and the number of stained vessels per mm^2 compared to PBS treatment.

Puiblication-2: "Effects of Pigment Epithelium Derived Factor on Traumatic Brain Injury"

In order to observe the PEDF expression pattern in post-traumatic rat brains, we measured mRNA levels of PEDF with RT-PCR at 4 hours, 1 day, 4 days, and 7 days after CCI (Fig. 2). We regarded all time points as separate experiments as different animals were used for each time point. Therefore, we compared only treatment and control, but not different time points with each other. PEDF mRNA levels in lesioned rat brains were significantly different at 1d, 4d, and 7d compared to sham operated rat brains.

To investigate whether PEDF infusion reduces contusion volume, we stained post-traumatic rat brains after PEDF or aCSF infusion with cresyl violet and calculated the contusion volume (Fig. 3). Statistical analysis revealed non-significant differences between lesioned brains treated with PEDF (5 and 10 µg/ml) and control groups.

To elucidate possible protective effects in post-traumatic brains after PEDF infusion, we investigated cell death and inflammatory processes in the lesion site. PEDF (5 and 10 µg/ml) significantly reduced the number of TUNEL-positive cells compared to control (Fig. 4). PEDF also reduced the number of ED1-positive cells (activated microglia) compared to aCSF (Fig. 5).

As PEDF has an influence on cell proliferation and self-renewal, we analyzed cell proliferation in posttraumatic brains after PEDF/aCSF infusion (Fig. 6), using BrdU labeling at the lesion site (Fig. 6A, B) and the main neurogenic regions; i.e. DG of hippocampus (Fig. 6A, C) and SVZ (Fig. 6A, D). In the lesion site, PEDF significantly decreased cell proliferation in lesioned brains compared to lesioned brains without pump. However, the number of BrdU-positive cells in the aCSF-treated group was not significantly different from the PEDF-treated groups (Fig. 6B). In DG of hippocampus, no significant effect of group attendance in the number of BrdU-positive cells was observed (Fig. 6C). In the SVZ, PEDF (5 and 10 µg/ml) increased the number of BrdU-positive cells compared to the lesioned brains without pump. We also detected a significant difference between aCSF and PEDF (10 µg/ml) treated lesioned brains (Fig. 6D).

Publication-3: "Influence of Pigment Epithelium-Derived Factor on Outcome after Striatal Cerebral Ischemia in the Mouse"

We investigated whether PEDF had an effect on stroke outcome using MCAO in mice. After 60 minutes MCAO, there was no significant difference, neither in lesion volume nor in edema between PEDF- and aCSF-infused post-ischemic mice (Fig. 2).

To evaluate exploratory locomotion and anxiety after MCAO, we performed open field test on day 7. We evaluated total distance traveled and time spent in the open center of the box. We observed no significant difference between PEDF and aCSF group (Fig. 3A). Pole test was performed on day 9 to assess motor function of mice after MCAO. We recorded the time that mice spent to turn completely head downwards (time-to-turn) and the total time it took to descend down (time down). Although there was no significant difference for time-to-turn between the treatment groups, time down significantly decreased in PEDF compared to aCSF group (Fig. 3B). Another measure of the influence of PEDF on motor function after MCAO is rotarod which we performed on day 10. We recorded the latency to fall off the accelerating rod. We observed no significant difference between the treatment groups (Fig. 3C). To evaluate spatial learning and memory after MCAO, Morris Water Maze (MWM) was performed. Before MWM test, we trained the animals for seven days prior to probe trial on day 21. During training, we measured the escape latency to reach the platform and total distance traveled. There was a significant effect of time (for PEDF between day 1 and day 5, for aCSF between day 1 and day 5, day 1 and day 6 as well as day 2 and day 6), but no significant effect of groups on escape latencies (Fig. 3D). For total distance traveled and escape latencies, we did not detect a significant effect between the treatment groups, but between the time points. For probe trial, we recorded the time in target quadrant. We did not find a significant difference between groups (Fig. 3E).

At day 21 after MCAO, we extracted mice brains and processed cryosections for BrdU immunohistochemistry to analyze cell proliferation in striatum, cortex, SVZ, and DG for CSF and PEDF. The number of BrdU-positive cells increased in the ipsilateral compared to contralateral striatum for both PEDF and CSF. However, there was no significant difference after PEDF infusion compared to CSF on each hemisphere. For cortex, PEDF but not CSF increased cell proliferation in the ipsilateral compared to contralateral hemisphere (Fig. 4B). For SVZ and dentate gyrus, there was neither a significant effect of hemispheres nor of treatment groups on the number of BrdU-positive cells (Fig. 4C). Regarding double stainings, we did not find any colocalization with NeuN. BrdU-positive cells were positive for GFAP or Iba1. But similar to the results

of overall proliferating cell number, there was no significant difference between CSF- and PEDF-treated groups (Fig. S1).
We performed TUNEL assay to investigate whether PEDF has an effect of cell death after MCAO. There was no significant difference in the number of TUNEL-positive cells between the groups (Fig. 5).

DISCUSSION

In my doctoral thesis, I investigated the influence of PEDF on three neurological disorders: i.e. peripheral nerve tumor (MPNST), brain trauma and stroke.
In publication-1, we showed that PEDF has significant effects on MPNST tumor cells, by influencing proliferation/viability, cell death, and vascularization, both *in vivo* and *in vitro*. As previously shown, NF1-derived MPNST cells secreted higher levels of PEDF compared to human-derived Schwann cells after adding axolema-enriched fraction to the culture. This is because PEDF secretion is inhibited in normal Schwann cells by axonal contact which is not the case in MPNST *in vivo*[40]. Furthermore, exogenous PEDF treatment promoted cell death in cancer cells *in vitro* and inhibited tumor growth *in vivo* by inducing endothelial apoptosis in different animal models[41, 42].
In our study, we supplemented additional exogenous PEDF to MPNST cells to see whether PEDF may trigger 1) different downstream pathways, 2) different mechanisms of action on proliferation, 3) differentiation, 4) death of tumor cells and 5) angiogenesis[1, 43]. We showed that *in vitro* and *in vivo* treatment with PEDF reduced cell viability in culture and the MPNST tumor burden in athymic nude mice by inhibiting angiogenesis and by inducing cell death in both tumoral and endothelial cells. Further studies are still needed to explore signaling pathways, mechanisms of action against tumor and endothelial cells as well as drug delivery strategies of PEDF before exploiting the potential of this anti-tumor molecule as a therapeutic agent.
In publication-2, we investigated the effects of PEDF in rats after TBI injury. *In vitro* and *in vivo* experiments have previously demonstrated the ability of PEDF to maintain survival of neurons after several brain insults such as acute brain injuries[28, 44]. We showed that PEDF mRNA levels in rats increased 7 days after trauma. This can be referred to as an endogenous compensation mechanism of the brain against neuronal degeneration to favor recovery after secondary damages, such as post-gliosis scar formation and neuroinflammation[45].

We aimed to see whether exogenous PEDF treatment has a protective effect on contusion volume after TBI. In a study using experimental stroke in rats, PEDF lowered lesion size as a neuroprotective factor[6] but we did not find a significant reduction in contusion volume in rat brains after PEDF treatment in our study. This might be because of the relatively low number of animals per group (n=4) that we used and the short end-point (8 days post-TBI), in which PEDF was not able to exert its effect on contusion volume. Despite the non-significant decrease in contusion volume, the number of TUNEL-positive cells significantly decreased after PEDF infusion compared to other control groups. PEDF was previously reported to reduce cell death[46-48]. Further studies have to be performed to investigate at which level PEDF interferes with the cell death cascade and/or when PEDF promotes its protective effects with respect to the onset of TBI.

As previously reported, PEDF does not only act on neurons or endothelial cells, but also on immune cells[27, 49]. We here demonstrated that PEDF infusion diminished the number of activated microglia around the lesion after CCI. Our finding is in line with the study of Acosta et al. which concluded that anti-inflammatory therapies might be promising during the chronic stage of TBI due to neurodegeneration after acute/chronic neuroinflammation[50].

Another known effect of PEDF is its stimulation on cell proliferation[28]. We therefore analyzed cellular proliferation after CCI at the site of the lesion and within neurogenic niches in the brain (SVZ and DG). PEDF infusion significantly reduced the number of BrdU-positive cells in the lesion. In the SVZ, but not in the DG, we found an increase in proliferating cells, as previously shown[28]. This spatiotemporal and cell type-specific effect of PEDF is based on its multifunctional feature which can be reflected in different ways in different cell types, such as microglia and stem cells. Nevertheless, the action mechanism of PEDF regarding target receptors and downstream pathways are not fully known so far.

In publication-3, we investigated whether PEDF can ameliorate behavioral outcome and influences cell death and cell proliferation after focal cerebral ischemia. We showed that in an ischemic lesion confined to the striatum, PEDF did not exert its previously described protective effects, and had no effect on lesion size, behavioral outcome, cell proliferation and cell death. In contrast to a previous study in transient MCAO[6], PEDF treatment did not reduce lesion volume and edema in our study.

The reason why PEDF did not exert its protective effects including behavioral outcome can be due to smaller size of lesion and edema or too small effects which were not detectable with the methods used in our study. Another explanation might be the route, duration and amount of PEDF administration. We only injected PEDF for 7 days and stopped injections two weeks prior to final euthanasia. This might not be sufficient to induce neuroprotection in our model. Other delivery techniques, such as striatal injections have been shown to be successful[51], as large molecules do not have to overcome the blood-brain barrier. However, we wanted to directly influence the neurogenic zone in our study. Furthermore, osmotic pump implantation was successfully used to deliver other trophic factors[52, 53].

To our knowledge, we were the first group to investigate the effect of PEDF on behavioral outcome and proliferation after MCAO. Although we did not see any effect of PEDF on cell proliferation in our model, intraventricular PEDF infusion was previously demonstrated to increase the number of BrdU-positive cells in the SVZ on both hemispheres of healthy animals[28]. In our study, we started PEDF infusion two days prior to MCAO and BrdU injection to see the effect of PEDF on cell proliferation after MCAO rather than on healthy brain. Therefore, we might not see the initial increase in the first two days in the number of BrdU-positive cells.

It was previously shown that cortical injury leads to an increase in cell proliferation in the SVZ, which might not clearly correspond to the increase in neurogenesis. The mechanisms of proliferation and migration of neuronal progenitor cells have not been fully understood so far[54]. Furthermore in our study, PEDF did not show protection against cell death in contrast to previous studies reporting this effect[55, 56].

Whereas PEDF reduced the extent of the lesion, cell death, inflammation and it modulated neurogenic processes after TBI in rats, it did not have an effect on lesion size, behavioral outcome, cell proliferation, or cell death after striatal ischemia in the MCAO mouse model. However, we cannot exclude that PEDF might work under different conditions in stroke. Therefore, further studies will elucidate the effect of PEDF treatment on cell proliferation and outcome in moderate to severe ischemic injury in the brain. Due to its inhibitory effects on the growth of human MPNST via induction of anti-angiogenesis and apoptosis, PEDF seems to be promising for future therapeutic purposes against MPNST.

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Selbständigkeitserklärung

„Ich, Menderes Yusuf Terzi, erkläre, dass ich die vorgelegte Dissertation mit dem Thema: “The role and influence of pigment epithelium-derived factor (PEDF) on peripheral nerve tumor, brain trauma and stroke” selbst verfasst und keine anderen als die angegebenen Quellen und Hilfsmittel benutzt, ohne die (unzulässige) Hilfe Dritter verfasst und auch in Teilen keine Kopien anderer Arbeiten dargestellt habe.“

Affidavit

I, Menderes Yusuf Terzi, certify under penalty of perjury by my own signature that I have submitted the thesis on the topic “The role and influence of pigment epithelium-derived factor (PEDF) on peripheral nerve tumor, brain trauma and stroke”. I wrote this thesis independently and without assistance from third parties, I used no other aids than the listed sources and resources.

All points based literally or in spirit on publications or presentations of other authors are, as such, in proper citations (see "uniform requirements for manuscripts (URM)" the ICMJE www.icmje.org) indicated. The sections on methodology (in particular practical work, laboratory requirements, statistical processing) and results (in particular images, graphics and tables) correspond to the URM (s.o) and are answered by me. My contributions in the selected publications for this dissertation correspond to those that are specified in the following joint declaration with the responsible person and supervisor. All publications resulting from this thesis and which I am author of correspond to the URM (see above) and I am solely responsible.

The importance of this affidavit and the criminal consequences of a false affidavit (section 156,161 of the Criminal Code) are known to me and I understand the rights and responsibilities stated therein.

BERLIN, den 19.11.2014

Anteilserklärung

Menderes Yusuf Terzi had the following share in the following publications:

Publication 1:

Maria Demestre*, **Menderes Yusuf Terzi***, Victor Mautner, Peter Vajkoczy, Andreas Kurtz, Ana Luisa Piña. Effects of pigment epithelium derived factor (PEDF) on malignant peripheral nerve sheath tumors (MPNSTs). J Neurooncol. 2013 Dec;115(3):391-9.

Individual contribution: 40% (* Equal contribution) – I did; preparation and experiments for histology and immunohistochemistry, countings and measurements of immunohistochemical data, preparation of figures and writing of paper.

Publication 2:

Menderes Yusuf Terzi, Pablo Casalis, Veronika Lang, Marietta Zille, Elisabeth Bruendl, Eva-Maria Stoerr, Alexander Brawanski, Peter Vajkoczy, Ulrich Thomale, Ana Luisa Piña. Effects of Pigment Epithelium Derived Factor on Traumatic Brain Injury. Restor Neurol Neurosci. 2015 Jan 1;33(1):81-93.

Individual contribution: %40 - I did; some of the experiments for histology and immunohistochemistry, countings and measurements of immunohistochemical data, preparation of figures and writing of paper.

Publication 3:

Marietta Zille, Arina Riabinska, **Menderes Yusuf Terzi**, Mustafa Balkaya, Vincent Prinz, Bettina Schmerl, Melina Nieminen-Kelhä, Matthias Endres, Peter Vajkoczy, Ana Luisa Piña. Influence of Pigment Epithelium-Derived Factor on Outcome after Striatal Cerebral Ischemia in the Mouse. PLoS One. 2014 Dec 3;9(12):e114595.

Individual contribution: %25 – I did; BrdU injections for immunohistochemistry, countings and measurements of immunohistochemical data, helped preparation of some figures and writing of paper.

Selected Publications

Electronic versions of the dissertations do not contain the original publications due to copy rights. The selected publications that are mentioned below can be reached from the respective DOI links.

Publication-1

"Effects of pigment epithelium derived factor (PEDF) on malignant peripheral nerve sheath tumors (MPNSTs)"

Authors: Maria Demestre, Menderes Yusuf Terzi, Victor Mautner, Peter Vajkoczy, Andreas Kurtz, Ana Luisa Piña.

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Publication-2

"Effects of Pigment Epithelium Derived Factor on Traumatic Brain Injury"

Authors: Menderes Yusuf Terzi, Pablo Casalis, Veronika Lang, Marietta Zille, Elisabeth Bruendl, Eva-Maria Stoerr, Alexander Brawanski, Peter Vajkoczy, Ulrich Thomale, Ana Luisa Piña.

Journal: Restor Neurol Neurosci. 33(1)…

Published: January, 2015

DOI: http://dx.doi.org/10.3233/RNN-140417

Publication-3

"Influence of Pigment Epithelium-Derived Factor on Outcome after Striatal Cerebral Ischemia in the Mouse"

Authors: Marietta Zille, Arina Riabinska, Menderes Yusuf Terzi, Mustafa Balkaya, Vincent Prinz, Bettina Schmerl, Melina Nieminen-Kelhä, Matthias Endres, Peter Vajkoczy, Ana Luisa Piña.

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Curriculum Vitae

Mein Lebenslauf wird aus datenschutzrechtlichen Gründen in der elektronischen Version meiner Arbeit nicht veröffentlicht.

List of Selected Publications for Publication Thesis:

Publication 1:

Maria Demestre*, **Menderes Yusuf Terzi***, Victor Mautner, Peter Vajkoczy, Andreas Kurtz, Ana Luisa Piña. Effects of pigment epithelium derived factor (PEDF) on malignant peripheral nerve sheath tumors (MPNSTs). J Neurooncol. 2013 Dec;115(3):391-9.

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Menderes Yusuf Terzi, Pablo Casalis, Veronika Lang, Marietta Zille, Elisabeth Bruendl, Eva-Maria Stoerr, Alexander Brawanski, Peter Vajkoczy, Ulrich Thomale, Ana Luisa Piña. Effects of Pigment Epithelium Derived Factor on Traumatic Brain Injury. Restor Neurol Neurosci. 2015 Jan 1;33(1):81-93.

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2. **Menderes Yusuf Terzi**, Pablo Casalis, Veronika Lang, Marietta Zille, Elisabeth Bruendl, Eva-Maria Stoerr, Alexander Brawanski, Peter Vajkoczy, Ulrich Thomale, Ana Luisa Piña. Effects of Pigment Epithelium Derived Factor on Traumatic Brain Injury. Restor Neurol Neurosci. 2014 November (Accepted).

3. Marietta Zille, Arina Riabinska, **Menderes Yusuf Terzi**, Mustafa Balkaya, Vincent Prinz, Bettina Schmerl, Melina Nieminen-Kelhä, Matthias Endres, Peter Vajkoczy, Ana Luisa Piña. Influence of Pigment Epithelium-Derived Factor on Outcome after Striatal Cerebral Ischemia in the Mouse. PLoS One. 2014 November (Accepted).

4. Stefanie Van Wagenen, Marion Kubitza, Markus Ackermann, **Menderes Yusuf Terzi**, Eva-Maria Stoerr, Joyce Tombran-Tink, Alexander Brawanski, Ana Luisa Pina. Age-related changes in PEDF and VEGF expression in pigmented and albino rat eyes. Molecular Vision. In preparation for submission.

Published contributions to academic conferences - Posters

1. Zille M., Riabinska A., Balkaya M., **Terzi M.Y.**, Prinz V., Schmerl B., Niemminen M., Endres M., Vajkoczy P., Dirnagl U., Pina A.L. Influence of Pigment Epithelium-Derived Factor on Neurogenesis and Angiogenesis after Cerebral Ischemia in the Mouse. Brain Meeting 2013. Shanghai, China. May 20-23, 2013.

2. Riabinska A., Cordell R., **Terzi M.Y.**, Zille M., Niemminen M.,Klohs J., Vajkoczy P., Dirnagl U., Pina A.L. Influence of Pigment Epithelium Derived Factor on Blood Brain

Barrier in Normal and Ischemic Brain. 10th Göttingen Meeting of the German Neuroscience Society. Göttingen, Germany. March 13-16, 2013.

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