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DISSERTATION

"Visualization of Cell Death after Experimental Stroke in the Mouse"

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Table of Contents

List of Abbreviations

AnxA5	Annexin A5
BBB	blood-brain barrier
BCA	bicinichoninic
CSF	cerebrospinal fluid
CT	computed tomography
DTPA	diethylene triamine pentaacetic acid
DTT	dithiothreitol
HPLC	high performance liquid chromatography
i.v.	intravenously
MCA	middle cerebral artery
MCAO	middle cerebral artery occlusion
NIRF	near-infrared fluorescence
PET	positron emission tomogrpahy
PI	propidium iodide
PS	phosphatidylserine
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
SPECT	single-photon emission computed tomography
TBR	target-to-background ratio
TTC	triphenyl tetrazolium chloride
TUNEL	terminal deoxynucleotidyl transferase-mediated 2'-deoxyuridine 5'-triphosphate-biotin nick end labeling

Abstract

Introduction: Cell death is one of the pathophysiological hallmarks after stroke. Markers to image death of brain cells *in vivo* are highly desirable. One of the candidate targets is phosphatidylserine (PS). Whereas PS is usually located on the inner leaflet of the plasma membrane bilayer facing the cytosol, it is translocated to the surface of the cell during cell death. This makes PS accessible for markers based on Annexin A5 (AnxA5) characterized by a high affinity and specific binding to PS. The aim of this thesis was to investigate whether AnxA5 can be used to specifically visualize cell death of brain cells after experimental stroke.

Methodology: In this thesis, AnxA5 was explored using three different approaches. First, we investigated a fluorescently labeled AnxA5 for *in vivo* and *ex vivo* near-infrared fluorescence imaging in experimental stroke in the mouse. We compared it to a non-binding control of AnxA5 and to other established cell death markers. In the second study, we evaluated a dual-labeled AnxA5 in the same animal model using single-photon emission computed tomography (SPECT), *ex vivo* activity measurements, and autoradiography. As a positive control, we used ethanol-induced cell death in the femur muscle. In the third study, we developed a long-circulating version of AnxA5, called XTEN-AnxA5. We examined its binding affinity using camptothecin-induced cell death in Jurkat T cells and specific accumulation inside chemotherapy-induced cell death in tumors.

Results: We showed that fluorescently labeled AnxA5 can be used to visualize cell death after experimental stroke *in vivo.* Only for functional, but not for non-binding AnxA5 control, increased signal intensities in the ipsilateral compared with contralateral hemisphere were found. AnxA5 specifically bound to dead or dying cells as confirmed by immunohistochemistry where the vast majority of cells were also positive for other cell death markers. However, we did not detect dual-labeled AnxA5 in the brain using SPECT, whereas it was observed in ethanol-induced cell death in the femur muscle. Our newly developed long-circulating XTEN-AnxA5 prolonged blood half-life to about 1 h and improved further imaging properties. We showed that XTEN-AnxA5 bound specifically to dead or dying cells in culture and displayed an increased accumulation inside the tumors compared to wild-type AnxA5.

Conclusions: Whereas AnxA5 seems to bind specifically to dead or dying cells, its applicability for brain imaging needs to be further investigated using compounds designed to overcome the blood-brain barrier and reach their target.

Abstrakt

Einleitung: Zelltod ist eines der pathophysiologischen Ereignisse nach Schlaganfall. Aus diesem Grund ist es erstrebenswert, Marker zur Bildgebung von Zelltod in vivo zu finden. Als mögliches Target kommt Phosphatidylserine (PS) in Frage. Während sich PS in gesunden Zellen auf der Innenseite der Plasmamembran dem Zytosol zugewandt befindet, wird es auf die Zelloberfläche transloziert, sobald eine Zelle stirbt. Auf diese Weise wird PS für Annexin A5 (AnxA5)-basierte Marker zugänglich, die dieses mit hoher Affinität und Spezifität binden. Ziel dieser Doktorarbeit war es, zu untersuchen, ob AnxA5 zur spezifischen Bildgebung von Zelltod nach experimentellem Schlaganfall eingesetzt werden kann.

Methodik: In dieser Arbeit wurde AnxA5 in drei verschiedenen Studien erforscht. In der ersten Studie untersuchten wir ein fluoreszenz-markiertes AnxA5 zur In-vivo- und Ex-vivo-Nah-Infrarot-Fluoreszenzbildgebung im Schlaganfallmodell der Maus. Dazu verglichen wir es mit einer nicht-bindenden AnxA5-Kontrollsubstanz und anderen etablierten Zelltodmarkern. In der zweiten Studie evaluierten wir ein zweifach markiertes AnxA5 im gleichen Tiermodell unter Verwendung von Einzelphotonen-Emissionscomputertomographie (SPECT), Ex-vivo-Aktivitätsmessungen und Autoradiographie. Zur Positivkontrolle diente Äthanol-induzierter Zelltod im Oberschenkelmuskel. Schließlich entwickelten wir in der dritten Studie ein lang zirkulierendes AnxA5, das so genannte XTEN-AnxA5. Wir untersuchten seine Bindungsaffinität mittels Camptothecin induziertem Zelltod in Jurkat-T-Zellen und die spezifische Anreicherung des Moleküls in Tumoren nach durch Chemotherapie induziertem Zelltod.

Ergebnisse: Wir konnten nachweisen, dass fluoreszenz-markiertes AnxA5 nach experimentellem Schlaganfall zur In-vivo-Zelltodbildgebung verwendet werden kann. Nur bei funktionellem, aber nicht bei der nicht-bindendem AnxA5-Kontrolle, konnten erhöhte Signalintensitäten in der geschädigten im Vergleich zur nicht geschädigten Gehirnhälfte gefunden werden. Tote oder sterbende Zellen wurden spezifisch von AnxA5 gebunden, was durch Immunhistochemie bestätigt wurde, wobei auch andere Zelltodmarker die Mehrzahl dieser Zellen markierten. Jedoch waren wir nicht in der Lage mit SPECT zweifach markiertes AnxA5 im Schlaganfall festzustellen, wohingegen es bei Äthanol-induziertem Zelltod im Oberschenkelmuskel nachgewiesen werden konnte. Unser neu entwickeltes lang zirkulierendes XTEN-AnxA5 verlängerte die Bluthalbwertzeit auf etwa eine Stunde und verbesserte weitere Bildgebungseigenschaften. Tote oder sterbende Zellen in Kultur wurden spezifisch von XTEN-AnxA5 gebunden und wir erreichten eine stärkere Anreicherung von XTEN-AnxA5 im Vergleich zu Wildtyp-AnxA5 in Tumoren.

Schlussfolgerung: Während AnxA5 scheinbar spezifisch an tote oder sterbende Zellen bindet, muss seine Eignung für Bildgebung im Gehirn unter Verwendung von Substanzen, die so ausgelegt sind, die Bluthirnschranke zu überwinden und das Zielmolekül zu erreichen, weiter untersucht werden.

Introduction

Stroke is a leading cause of death and disability in developed countries.[1] One of the main hallmarks of a stroke as well as of many other neurological and neurodegenerative diseases is cell death.[2] Until now, the molecular mechanisms underlying cell death in the brain are only partially understood.[2, 3] A therapy reducing cell death after stroke is still pending.

Current research for cell death in models of neurological diseases is limited because most of the data are obtained at a single time point at which the experiment must be terminated in order to sacrifice the animals to perform histological or biochemical analysis of the specimen.[4] Studying the distribution and dynamics of cell death cascades in the same animal is thus restricted. To this date, an imaging marker for specific noninvasive visualization of molecular targets involved in cell death after stroke has not yet been established. Such a marker is highly desirable. It would allow for monitoring the reduction of cell death after therapy in stroke and other diseases.

A candidate target for such a marker is phosphatidylserine (PS). In a healthy cell, PS resides on the inner leaflet of the plasma membrane. It is translocated to the surface of the cell during cell death. The Ca^{2+}-dependent binding protein Annexin A5 (AnxA5) binds specifically and with high affinity to PS and to other anionic phospholipids, which reflects its biological role as an anticoagulant. Imaging of markers based on AnxA5 has already been successfully established and extensively studied in atherosclerosis,[5] cancer,[6, 7] and myocardial infarction.[8]

Interestingly, two patients with acute stroke receiving ^{99m}Tc-HYNIC-AnxA5 (99mTc is conjugated via amine bonds to AnxA5) were reported to show uptake of AnxA5. It correlated with regions of restricted diffusion on magnetic resonance imaging (MRI). The authors also investigated the distribution of the same marker in response to Anti-Fas Ligand antibody treatment after 2 h of middle cerebral artery occlusion (MCAO), an experimental stroke model in rodents. They showed that infarct size and number of dead cells correlated well with tracer uptake.[9] The same group reported that eight out of twelve stroke patients displayed increased uptake of AnxA5 corresponding to the infarct region identified by computed tomography (CT).[10] Furthermore, AnxA5 was successfully used to investigate the protective effects of minocycline treatment in a permanent MCAO model in mice. The authors showed that minocycline treatment decreased AnxA5 uptake significantly in lesioned hemispheres. The uptake signal correlated well with infarct volumes, too.[11]

Objective

Our hypothesis was that AnxA5 can be used to specifically visualize cell death of brain cells after experimental stroke. Therefore, we first used a fluorescently labeled AnxA5 for *in vivo* near-infrared fluorescence (NIRF) imaging in MCAO mice to investigate binding specificity compared to other cell death markers. However, NIRF imaging is not applicable to visualize cell death in the brain of patients. For future translation of our results into the clinic, in the second study, we investigated whether a dual-labeled AnxA5 is suitable for single-photon emission computed tomography (SPECT) in MCAO mice. To overcome the blood-brain barrier (BBB) is a major challenge in delivery of compounds to the brain. In the third study, we consequently developed a long-circulating version of AnxA5, which should allow for improved imaging of target tissues.

Methodology

All materials and methods used in this thesis are described in the publications listed in the appendix. In this section, I summarized the main materials and methods related to key findings.

Animals and Ethical Statement

We performed all procedures in accordance with the German animal welfare laws (Landesamt fuer Gesundheit und Soziales Berlin). Local guidelines for premature euthanasia were followed when animals showed signs of distress in our daily health check. Animals were housed under standard conditions with free access to food and water. We randomly assigned animals to experimental groups and investigators were blinded to the experimental groups while analyzing the results.

Labeling of AnxA5 with Cy5.5 for NIRF Imaging

Human recombinant AnxA5 (from Chris P Reutelingsperger, Maastricht University, Maastricht, The Netherlands) was labeled with Cy5.5 (Cy5.5-N-hydroxysuccinimide ester, PA 15601, Amersham Biosciences, Buckinghamshire, UK, Em_{Max}=694 nm, Ex_{Max}=675 nm) as previously described.[12] We labeled functional AnxA5, which retained its affinity for PS, by using about one mole of Cy5.5 per mole of protein. In contrast, nonfunctional AnxA5 was prepared with two moles or more of dye per mole of protein leading to a complete loss of affinity for PS. We diluted both AnxA5 preparations to a volume of 150 µl in saline and 7 nmol of Cy5.5 per mouse (about 0.3 mg of protein for functional and 0.1 mg of protein for nonfunctional AnxA5).

Labeling of HIS-cys-AnxA5-AF568 with $^{99m}Tc(CO)_3$ for SPECT Imaging

Synthesis of HIS-cys-AnxA5-AF568 and HIS-cys-M1234-AnxA5-AF568 was performed by Chris P Reutelingsperger as previously described.[13] We radioactively labeled 70 µg of both proteins with $Tc(CO)_3^+$ as

described in detail in publication 2. A total yield of 70.99±8.88% for $^{99m}Tc(CO)_3$-HIS-cys-AnxA5-AF568 and 77.21±3.03% for $^{99m}Tc(CO)_3$-HIS-cys-M1234-AnxA5-AF568 in 0.9% saline (total volume of 100-150 µl per mouse) was achieved.

Expression and Purification of XTEN-AnxA5

The cDNA of the human AnxA5 (NP 1145.1) was joined with an XTEN sequence of 288 amino acids (XTEN288) to obtain the fusion protein.[14] To allow for labeling with metal chelators for radioactive tracers and thiol reactive fluorophores, we added one cysteine on the N-terminus. AnxA5 has an endogenous cysteine at position 316, which would lead to unspecific labeling. It was therefore mutated to serine. In addition, we inserted two stop codons at the C-terminus to prevent C-terminal tag expression of the plasmid. XTEN-AnxA5 was produced in heat shock-competent E.coli BL21(DE3) Gold Cells (Agilent Technologies, Boeblingen, Germany). We purified XTEN-AnxA5 using anionic exchange and hydrophobic interaction columns and assessed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and high performance liquid chromatography (HPLC) as described in the publication 3.

Labeling of XTEN-AnxA5 with DTPA and 6S-IDCC for NIRF Imaging and $^{111}In^{3+}$ for SPECT Imaging

To reduce potential cysteine dimers at the N-terminus, we incubated XTEN-AnxA5 with dithiothreitol (DTT, Sigma-Aldrich, Steinheim, Germany) as described in publication 3. Maleimide-diethylene triamine pentaacetic acid (Maleimide-DTPA, CheMatech, Dijon, France) or maleimide-6S-IDCC (Mivenion GmbH, Berlin, Germany, Em_{Max}=695nm, Ex_{Max}=675 nm) was added to the protein solution in 20-fold molar excess. We performed labeling of AnxA5 (as a control compound) accordingly (6S-IDCC-AnxA5). Final concentrations of XTEN-AnxA5-DTPA, 6S-IDCC-XTEN-AnxA5, and 6S-IDCC-AnxA5 were determined using bicinchoninic acid (BCA) protein assay as described in more detail in publication 3.

For labeling with $^{111}In^{3+}$, we mixed 121.2 µg of XTEN-AnxA5-DTPA with buffer (270 mM NaOAc/ HOAc, 79 mM Gentisin, pH 5.0) and added $^{111}InCl_3$ solution (0.02 M HCl, 291 MBq, 157.3 ng indium) as described in publication 3. This yielded 71% XTEN-AnxA5-^{111}In in buffer solution.

Induction of Cell Death In Vitro and Flow Cytometry

To investigate binding of XTEN-AnxA5 in study 3, human T-cell leukemia cells (Jurkat cells, Leibniz Institute DSMZ – German collection of Microorganisms and Cell Culture) were cultivated as described in publication 3. We incubated the cells with 9 mM camptothecin (Sigma-Aldrich, Steinheim, Germany) for 5 h to induce cell death. Then, they were harvested and incubated with 6S-IDCC-XTEN-AnxA5 or wild-type FITC-AnxA5 (FITC Annexin V Apoptosis Detection Kit I, BD Biosciences, Heidelberg, Germany) as described in more detail in publication 3. We performed flow cytometric measurements using FACSCalibur

flow Cytometer (BD Biosciences, Heidelberg, Germany) at 488 nm for FITC-AnxA5 and 633 for 6S-IDCC-XTEN-AnxA5. We analyzed the data using FlowJo 8.7 (Tree Star Inc., Ashland, USA).

Middle Cerebral Artery Occlusion

In study 1 and 2, we induced transient MCAO in 8-10-week-old male C57BL6/N mice (Bundesinstitut fuer Risikoforschung for study 1 or Research Institute for Experimental Medicine, Charité, Berlin, Germany for study 2). We inserted an 8-0 nylon monofilament (Suprama, Berlin Germany) coated with silicon resin (Heraeus Kulzer, Hanau, Germany) for study 1 or silicone rubber-coated, 170 µm thick monofilament (Doccol Corporation, Redlands, USA) for study 2 via the internal carotid artery as described by Engel and colleagues.[15] Anesthesia was performed using isoflurane (2% for induction and 1.5% for maintenance) in 70% N_2O and 30% O_2 via a facemask. After 1 hour, mice were re-anesthetized and the filament removed to allow for reperfusion of the MCA. We treated the mice with Lidocain (study 1) or Bupivacain (study 2) as a local anesthesia after surgery. We measured their body temperature and kept it constant between 37.0 and 37.5 °C with a heating pad during surgery and ischemia. After surgery, the animals woke up in a warming cage and stayed there for around 2 h.

Ethanol-Induced Cell Death in the Femur Muscle

As a positive control in study 2, we induced ethanol-induced cell death by injecting 100 µl of 100% ethanol into the left femur muscle of 10-week-old male C57BL6/N mice (Research Institute for Experimental Medicine, Charité, Berlin, Germany) as previously described.[16] Mice were anesthetized as specified for MCAO. We allowed the mice to recover for 19 h and subcutaneously injected of buprenorphin (0.05 mg/kg) every 8 h for pain relief.

Tumor Xenograft Model

For study 3, $1x10^6$ EL4 cells (mouse lymphoma cell line) in 100 µl DEMEM (Sigma-Aldrich, Steinheim, Germany) were inoculated subcutaneously into both flanks of female immunodeficient nude mice (Charles River Laboratories, Sulzfeld, Germany). We allowed the tumors to grow for about one week until a size of 5 to 10 mm. Mice received intravenous (i.v.) injections of 19 mg/kg etoposide and 25 mg/kg cyclophosphamide to induce cell death in the tumor.

NIRF Imaging and Propidium Iodide Injection

For study 1, Cy5.5-AnxA5 was injected i.v. into the tail vein at 48 h after MCAO (n=13 for functional, n=8 for nonfunctional Cy5.5-AnxA5). NIRF Imaging was performed 4 h (n=8 for functional, n=5 for nonfunctional) and 8 h (n=5 for functional, n=3 for nonfunctional) after injection. Three mice per group of those mice imaged at 4 h after injection also received intraperitoneal injections of 20mg/kg propidium iodide (PI,

Molecular Probes, Eugene, OR, USA, diluted in 150 µl saline). PI stains cells with compromised plasma membrane integrity. We anesthetized the mice with intraperitoneal injections of a 5% chloralhydrate solution (200 mg/kg, Merck, Darmstadt, Germany). We depilated the skin of the head. For study 3, 6S-IDCC-XTEN-AnxA5 or 6S-IDCC-AnxA5 was injected i.v. into tumor xenograft mice (n=4 per group) and NIRF imaging performed 3 h later.

We performed NIRF imaging with a custom-made imager using fluorescence reflectance imaging mode.[17] Images were processed and analysis was performed as previously described.[17] Briefly, we normalized images, corrected illumination inhomogeneities and subtracted superficial fluorescence from dust or photons from cosmic rays. We manually outlined tegions of interest (ROIs) on both hemispheres for MCAO mice or whole organs and hotspots in tumors for the murine xenograft model. Average fluorescence intensities of all pixels within the ROI were calculated. We obtained target-to-background ratios (TBRs) by calculating the ratio between ipsi- and contralateral hemisphere.

SPECT/CT Imaging

For study 2, anesthetized mice received i.v. injections of 96.43±30.00 MBq $^{99m}Tc(CO)_3$-HIS-cys-AnxA5-AF568 at 24 (n=5), 48 (n=3) or 72 (n=5) or 142.45±23.09 MBq $^{99m}Tc(CO)_3$-HIS-cys-M1234-AnxA5-AF568 at 72 h (n=3) (±2.0) h after reperfusion after MCAO. For ethanol-induced cell death in the left femur muscle, i.v. injections were performed at 19 (±.5) h after injury onset (n=5 for $^{99m}Tc(CO)_3$-HIS-cys-AnxA5-AF568 and n=3 for $^{99m}Tc(CO)_3$-HIS-cys-M1234-AnxA5-AF568). One mouse that received $^{99m}Tc(CO)_3$-HIS-cys-M1234-AnxA5-AF568 at 72 h after reperfusion did not survive the injection.

For study 3, four- to five-week old female Balb/c mice (Charles River Laboratories, Sulzfeld, Germany) were anesthetized and received i.v. injections of ~100 MBq of ^{111}In-XTEN-AnxA5 (n=5) in order to calculate its blood half-life. Sequences for SPECT/CT imaging are described in more detail in publication 2 and 3.

Ex Vivo Biodistribution Measurements and Autoradiography

After SPECT imaging, we sacrificed the mice by decapitation under deep anesthesia. We determined organ biodistribution in an activity meter ISOMED 2010 (MED Nuklear-Medizintechnik Dresden GmbH, Dresden, Germany). The percentage of injected dose (% ID) and % ID/organ weight (%ID/g) was calculated. Brains of MCAO animals were immediately snap frozen in ice-cold methyl butane. We cut 20 µm-thick coronal cryosections at a Cryostat (MTC, SLEE medical GmbH, Mainz, Germany) at interaural positions 6.6, 5.3, 3.9, 1.9, and 0 mm. One series of sections was incubated overnight on a technetium-sensitive imaging plate (Fuji Imaging plate; BAS-IP TR 2025, Duesseldorf, Germany) and digitized using

Bio-Imaging Analyzer BAS 5000 (Fuji, Duesseldorf, Germany). We performed ROI analysis to obtain TBRs using ImageJ v.1.45 (http://rsb.info.nih.gov/ij/index.html) as follows: For MCAO animals, we calculated ratios between areas of the lesioned striatum and cortex on the ipsilateral hemisphere and mirrored contralateral areas for MCAO, both corrected by background subtraction. For animals undergoing ethanol-induced cell death in the left femur muscle, we measured ratios between ipsilateral muscle and contralateral muscle also corrected by background subtraction.

Infarct Volumetry

For study 1, we used brains of the mice not injected with PI for infarct staining with triphenyl tetrazolium chloride (TTC, Sigma-Aldrich, Hamburg, Germany). For more details, see publication 1. For infarct volumetry of cryosections, hemalaun staining was performed as described in publication 1 and 2. We quantified the area of infarction by ImageJ as defined by the colorless area in TTC staining and lower overall cell density in hemalaun staining. We applied edema correction as follows: 'indirect' infarct volume = volume of the contralateral hemisphere minus volume of the non-infarcted ipsilateral hemisphere.

Immunohistochemistry and Microscopy

For study 1, brains of MCAO mice receiving PI injections were snap-frozen and cut as specified above. Cy5.5 and PI fluorescence were examined before terminal deoxynucleotidyl transferase-mediated 2'-deoxyuridine 5'-triphosphate-biotin nick end labeling (TUNEL) immunohistochemistry as described in detail in publication 1. Finally, we performed hemalaun staining on the same sections. We calculated the percentage of cells immunoreactive for none, one, two, or all three markers inside the cortical and striatal lesions by manual counting of three sections from each mouse.

For study 2, as AnxA5 was labeled by AF568, the sections were also investigated under fluorescent microscopy using a Leica DMRE microscope (Leica Microsystems Jena GmbH, Jena, Germany).

For study 3, 10-µm thick slices of tumors were cut on a cryostat. We investigated the distribution of 6S-IDCC-XTEN-AnxA5 and 6S-IDCC-AnxA5 under a fluorescence microscope (Zeiss Observer.Z1, Carl Zeiss AG, Jena, Germany) with Hoechst33258 nuclear counterstain (Sigma-Aldrich, Steinheim, Germany). We performed Caspase-3 staining, for more details see publication 3.

Statistics

We tested normality using Kolmogorov-Smirnov test and variance homogeneity using Levené test. For normally distributed data where variances were not homogenous across groups and more than two groups were analyzed, Kruskal-Wallis test was used followed by post hoc Mann-Whitney U test with α-correction according to Bonferroni to adjust for the inflation of type I error due to multiple testing (fami-

lywise error rate). When data were distributed normally and variances were homogenous across groups, independent t test was performed in case of two groups and one-way ANOVA in case of more than two groups. For target-to-background ratios of MCAO animals vs. infarct volumes, we performed Spearman's Rank Order correlation because variances were not homogenous across groups. $p<.05$ was considered to be statistically significant. For Kruskal-Wallis test performed followed by Mann-Whitney U test, $p=.05/k$ was used, with k as the number of single hypotheses. We performed statistical analyses with SPSS v.19.0 or Prism v5 (GraphPad Software).

Results

The results of the studies are described in detail in the publications listed in the appendix. In this section, I summarize the main findings of the publications.

Study 1: "Visualization of cell death in mice with focal cerebral ischemia using fluorescent annexin A5, propidium iodide, and TUNEL staining"

We were able to show that death of brain cells after 60-min MCAO in mice can be specifically visualized noninvasively using NIRF imaging of Cy5.5-AnxA5.

At 48 h after reperfusion, we injected MCAO mice with functional and nonfunctional (control) Cy5.5-AnxA5. Mice receiving functional Cy5.5-AnxA5 showed significantly higher TBRs 4 and 8 h after injection compared to mice receiving nonfunctional Cy5.5-AnxA5 (1.16±.07 vs. 1.02±.02 at 4 hours for functional vs. nonfunctional Cy5.5-AnxA5, Student's t-test, $p=.002$; 1.18±.08 vs. 1.01±.01 at 8 hours for functional vs. nonfunctional Cy5.5-AnxA5, $p=.008$, Figure 2). *Ex vivo*, areas with high AnxA5 fluorescence intensity corresponded well with the lesion site as delineated by TTC staining (Figure 3). We also observed low fluorescence intensities in the infarct area in animals receiving nonfunctional AnxA5 due to passive leakage of the molecule through a disturbed BBB (Figure 1 and 3).

Furthermore, for the first time, we compared the distribution of i.v. injected functional as well as nonfunctional AnxA5, PI, and subsequent TUNEL staining (Figure 5). We found that about 59% and 53% of the cells were positive for AnxA5 in striatum and cortex, respectively. More than 80% of these cells also showed a signal for PI and TUNEL (striatum: 84%, cortex: 89%). The majority of the remaining cells positive for AnxA5 was PI-positive. There were only a few AnxA5-positive cells without any other label. However, a considerable fraction of cells was positive for PI and TUNEL or PI alone (striatum: 18%, cortex: 16%) (Table 1). Interestingly, we noted a strong immunoreactivity for AnxA5 and PI, but not TUNEL in the choroid plexus inside the ventricles (Figure 4).

Study 2: "**A dual-labeled Annexin A5 is not suited for SPECT imaging of brain cell death in experimental murine stroke"**

In this study, we demonstrated that dual-labeled AnxA5 is not suited to visualize death of brain cells in a 60-min MCAO in mice using SPECT.

MCAO mice were injected i.v. with $^{99m}Tc(CO)_3$-HIS-cys-AnxA5-AF568 (HIS-AnxA5) at 24, 48, or 72 h or the nonfunctional $^{99m}Tc(CO)_3$-HIS-cys-M1234-AnxA5-AF568 (M1234, negative control) at 72 h after reperfusion. Immediately after and then every 30 min until 3.5 h after injection, we performed SPECT imaging. We did not detect any AnxA5 SPECT signal in the brain at any time point investigated (Figure 1A). We showed that the biodistribution of both AnxA5s was as expected with the highest uptake in kidneys (39.93±14.39% of injected dose for HIS-AnxA5 (n=13) vs. 53.29±6.73% for M1234 (n=2)) and the liver (11.40±3.57% for HIS-AnxA5 vs. 6.26±3.11% for M1234). To investigate whether a signal was not detected due to sensitivity issue, we performed *ex vivo* AnxA5 autoradiography. Interestingly, we found a spotty distribution of AnxA5 inside the brain, even sometimes on the contralateral side, and at the rim of the slice. A significant difference in median target-to-background ratios between the groups and compared to control was not observed (Figure 1B). Data were normally distributed (Kolmogorov-Smirnov test, Z=.827, p=.501), but variances were not homogenous across groups (Levené test, F(3,11)=7.725, p=.005). Median target-to-background ratios were 1.22 for 24 h (n=5), 1.15 for 48 h (n=3), 2.32 for 72 h (n=5), and 1.78 for control at 72 h (n=2). A significant effect in the omnibus test was observed (Kruskal-Wallis test, $\chi^2(3,N=15)=9.292$, p=.026, $\eta^2=.664$). Further post hoc analysis with Mann-Whitney U and Bonferroni correction revealed no significant differences between the groups at α=.05/6; corrected α=.0083 (p=.881 for 24 vs 48 h, p=.009 for 24 vs. 72 h, p=.053 for 24 h vs. control, p=.051 for 48 vs. 72 h, p=.236 for 48 vs. control, p=.439 for 72 h vs. control, two-tailed). Infarct volumetry using hemalaun staining did not reveal any differences between the groups (Figure 1C). Data were normally distributed (Kolmogorov-Smirnov test, Z=.593, p=.874) and variances homogenous across groups (Levené test, F(3,11)=.908, p=.468). Mean infarct volumes were 62.36±30.62 for 24 h (n=5), 47.21±31.18 for 48 h (n=3), 63.10±19.65 for 72 h (n=5), and 72.96±8.17 for control at 72 h (n=2). No significant effect of group attendance was observed (one-way ANOVA, F(3,11)=.445, p=.725, $\omega^2=.108$). We ran a Spearman's Rank Order correlation to determine the relationship between TBRs and infarct sizes for all but the M1234 control animals. We did not find a correlation between the two ($r_s(11)=-.113$, p=.714).

In contrast, we were able to detect cell death in the model of ethanol-induced cell death in the femur muscle. 19 h after EtOH-induced cell death in the femur muscle, we injected mice i.v. with HIS-AnxA5 or M1234 control and performed SPECT imaging immediately after and every 30 min until 3.5 h after injec-

tion. We found an AnxA5 signal in the ipsilateral but not in the contralateral muscle in mice receiving HIS-AnxA5. Mice receiving M1234 control did not display any signal *in vivo* (Figure 2A). Again, we observed high uptake in kidneys (47.00±1.51% of injected dose for HIS-AnxA5 (n=5) vs. 45.90±6.01% for M1234 (n=3)) and liver (13.23±4.45% for HIS-AnxA5 vs. 7.96±.35% for M1234). In *ex vivo* autoradiography, mean TBRs between ipsilateral and contralateral muscles were significantly higher in mice receiving HIS-AnxA5 compared to M1234 control (Figure 2B). Data were normally distributed (Kolmogorov-Smirnov test, Z=.577, p=.894) and variances homogenous across groups (Levené test, F(1,6)=2.007, p=.206). Mean target-to-background ratios of $^{99m}Tc(CO)_3$-HIS-cys-AnxA5-AF568 were significantly higher (3.86±1.12, n=5) compared to M1234 control (1.84±.47, n=3) (independent t test, t(6)=2.898, p=.027, r=.762, Figure 2C).

Furthermore, we used the fluorescent label AF568 of the AnxA5 marker to perform fluorescence microscopy. We only found AnxA5 immunoreactivity in the femur muscle undergoing ethanol-induced cell death (Figure 3).

Study 3: "XTEN-Annexin A5: XTEN Allows Complete Expression of Long-Circulating Protein-Based Imaging Probes as Recombinant Alternative to PEGylation"

The blood half-life of the AnxA5 used in study 2 is only about 20 min in mice. This limits its ability to reach the brain. Therefore, we developed a long-circulating version of AnxA5. In this study, we demonstrated that XTEN-AnxA5 has a blood half-life of about 1 h in mice and provided a first proof-of-concept for its ability to bind to dead or dying cells in a tumor model in mice.

We expressed XTEN-AnxA5 fusion protein in E. coli and purified the protein using anionic exchange and hydrophobic interaction columns (Figure 1). We confirmed purity of 95% using SDS-PAGE gel and HPLC. Mass spectroscopy revealed a mass of 62.48 kDa, which was within the typical range of the calculated mass of 62.37 kDa. Using Western blotting, we showed that antibodies directed against wild-type AnxA5 bound to XTEN-AnxA5 as well (Figure 2).

We then labeled XTEN-AnxA5 with the NIRF dye maleimide-6S-IDCC for fluorescence microscopy and NIRF or maleimide-DTPA for SPECT imaging. First, we investigated binding of maleimide-6S-IDCC-XTEN-AnxA5 compared to wild-type FITC-AnxA5 in a cell culture of camptothecin-treated Jurkat T cells revealing a selective and a similar binding pattern of both AnxA5s. In addition, we showed that calcium-dependent binding affinity was similar for both AnxA5s as examined with a calcium titration assay (Figure 3). Then, ^{111}In-DTPA-XTEN-AnxA5 was injected i.v. into healthy mice and allowed to circulate. The calculated blood half-life was 63.3 ± 4.4 min, confirmed by *ex vivo* biodistribution data (Figure 4). We found

high uptake in spleen, liver, and kidneys (Figure 5). Lastly, we evaluated whether the prolonged blood half-life of XTEN-AnxA5 improves target accumulation. Therefore, we implanted tumor xenografts (EL4 cell line) into the flanks of immunodeficient nude mice. Tumors were treated with etoposide and cyclophosphamide for tumor apoptosis. One day later, mice received i.v. injections of NIRF-labeled XTEN- or wild-type AnxA5. We performed NIRF imaging at 3 h after injection and demonstrated a significantly higher uptake of XTEN-AnxA5 compared to wild-type AnxA5 (2.37 vs. .986 arbitrary units, two-tailed Wilcoxon-Mann-Whitney U test, p=.03, n=4 per group). *Ex vivo* fluorescence microscopy confirmed this result. All tumors displayed similar levels of cell death as investigated by caspase-3 immunohistochemistry (Figure 6).

Discussion

The objective of this thesis was to investigate whether AnxA5 is suitable to specifically visualize cell death in the brain after experimental stroke.

In the first study employing Cy5.5-labeled AnxA5 for NIRF imaging after MCAO in mice, we demonstrated high specificity of this marker to dead or dying cells inside the brain both *in vivo* and *ex vivo*. This was strongly supported by a co-registration of AnxA5-positive cells with other cell death markers, i.e. TUNEL and PI. We also showed that a nonfunctional version of AnxA5 had significantly lower TBRs. This might be attributed to unspecific leakage of markers across the disrupted BBB into the brain parenchyma.

Our first study had three major limitations: (1) NIRF imaging cannot be used in stroke patients, (2) the NIRF signal cannot be quantified due to absorption and reflection inside the tissue, and (3) an estimation of the amount of unspecific leakage vs. binding of the marker is not possible. At that time, a number of AnxA5 probes already existed that were used for different imaging modalities apart from optical imaging, including magnetic resonance imaging,[18, 19] ultrasound,[20] and nuclear imaging.[16, 21-25] As compared to MRI and CT, positron emission tomography (PET) and SPECT imaging offer a higher sensitivity. This is of major importance especially for brain imaging where molecular delivery, and thus signal intensity, is largely limited by the BBB. However, small animal imaging PET and SPECT scanners have a lower spatial resolution, but a combination with MRI or CT allows for sufficient anatomical localization of tracer uptake.[26] Furthermore, SPECT can be used to discriminate between multiple radioactive labels allowing for the estimation of the amount of unspecific leakage compared to functional binding. Overall, SPECT imaging seemed to be the most suitable imaging modality to assess AnxA5 for future translation into the clinic. In a first series of studies, ^{99m}Tc-HYNIC-AnxA5 had been used to visualize cell death in a small number of stroke patients and in a mouse model of focal cerebral ischemia using SPECT imaging. However, specificity of AnxA5 was not assessed in these studies.[9-11]

In our second study, we used a so-called 'second generation' AnxA5, namely $^{99m}Tc(CO)_3$-HIS-cys-AnxA5-AF568, for SPECT imaging of cell death after experimental stroke. The derivative comprised a cysteine residue at its concave site. This enables site-specific conjugation of different radiometal binding ligands via sulfide formation through thiol chemistry. In consequence, the radioisotope cannot affect the binding properties of AnxA5 because it is located outside its binding region.[13] As a linker for radioactive labeling, we attached a HIS-tag with six histidine residues to the N-terminus of the protein. In a previous study, we demonstrated that $^{99m}Tc(CO)_3$-HIS-cys-AnxA5 has improved *in vivo* affinity, distribution in the body, stability, and radiolabeling yields compared to the 'first generation' ^{99m}Tc-HYNIC-AnxA5.[13] In order to be able to detect the tracer using fluorescent microscopy, we also conjugated the marker to AF568.

Astonishingly, we were not able to find an accumulation of dual-labeled AnxA5 inside the brain using SPECT imaging after experimental cerebral ischemia. At first, we speculated that sensitivity might be an issue. However, in *ex vivo* activity measurements, we detected high activity only in the kidneys and the liver confirming SPECT imaging. Similar biodistributions have been reported previously[16, 25, 27] with accumulation in the kidneys to be considered to stem from a strong PS expression in the cortex of the kidneys.[28] We did not find a difference in TBRs using *ex vivo* autoradiography as a more sensitive detection method, either. To rule out that $^{99m}Tc(CO)_3$-HIS-cys-AnxA5 did not bind anymore to dead or dying cells, we employed a positive control, i.e. a model of ethanol-induced cell death in the femur muscle. We detected binding only in the injured muscle both in SPECT imaging and *ex vivo* autoradiography.

A second reason why AnxA5 was not detectable inside the brain might have been that it did not overcome the BBB. Although it has previously been shown that the BBB is disrupted after cerebral ischemia,[29-31] this does not necessarily mean that every molecule can enter the brain. It rather depends on physiochemical properties and on the molecular weight of the marker.[32] However, both AnxA5 molecules used in study 1 and 2 did not differ much in their molecular weight. In contrast, there is a larger difference in terms of blood half-life with about 7 min for wild-type AnxA5 and about 20 min for the dual-labeled AnxA5 used here.[33] Prolonging blood half-life should increase the probability for a marker to reach its target, which does not seem to be effective for the AnxA5 investigated here. Whether the half-life was not long enough or whether Cy5.5-AnxA5 has other characteristics allowing for BBB crossing was not evaluated in this study.

Interestingly, we found that AnxA5 was distributed in a spotty manner with high accumulation inside the ventricles and at the rim of the sections. This is consistent with our observations in the first study. We think that AnxA5 might have drained into the cerebrospinal fluid (CSF). This is possible at two sites: the blood-CSF barrier at the choroid plexus and the outer CSF-brain barrier between the CSF-filled sub-

arachnoid space and overlying structures. It has been demonstrated that the choroid plexus is not restrictive for large molecules and that proteins equilibrate in the stromal compartment filling the basolateral intercellular spaces up to the tight junction complexes.[34] In one of the studies in stroke patients, biodistribution of AnxA5 in control patients also resulted in an accumulation inside the venous sinuses and choroid plexus.[10]

In the third study, we developed a long-circulating version of AnxA5 to increase its probability to reach the brain and to be able to attach different radioisotopes. The latter then makes it possible to overcome the third limitation described above, namely to be able to estimate the amount of signal coming from unspecific leakage into the brain parenchyma compared to functional binding of the marker. This is only possible when more than one kind of radioisotope can be attached to the molecule because both nonfunctional and functional protein can then be injected into the same animal and distinguished by the different gamma photon energies of the two nuclides.[35]

We therefore designed an XTEN-AnxA5 fusion protein, which can be used for labeling with multiple nuclides when coupled to DTPA. The XTEN sequence comprised 288 amino acids which lead to an increase in molecular weight of AnxA5 from 36 to 62 kDa. As a first step, we confirmed that XTEN-AnxA5 still bound to dead or dying cells in camptothecin-treated Jurkat T cells and that binding was even enhanced compared to wild-type AnxA5. Using nuclear imaging in healthy mice, we demonstrated an extended blood half-life of about 1 h. Blood clearance of XTEN-AnxA5 by the kidneys was decreased, whereas it was increased by spleen and liver, similar to what has previously been reported for PEGylated AnxA5.[36]

In PEGylation, polyethylene glycol (PEG) polymer chains are covalently attached to another molecule. PEGylation is an alternative way to increase blood half-life of proteins. However, there are several disadvantages of PEG relevant in terms of clinical use: (1) While XTEN serves as a tag, an additional synthesis and purification step is needed for PEGylation. (2) Particularly for amine-directed coupling, PEGylation can result in partial inactivation of the protein,[36] a complication to which AnxA5 is especially susceptible.[37] (3) Mixtures of proteins having no, one, or multiple-coupled PEGs occur that are hard to separate.[36] (4) It was also reported that PEGylated protein complexes may be cleaved metabolically. This results in PEG moieties circulating in the bloodstream and tissues.[38] PEG in not biodegradable and thus might be toxic over time.[39] In contrast, XTEN is genetically developed with a defined size, and made of biodegradable amino acids. XTEN has been shown to have low immunogenicity in animals.[14]

We realized that the experimental stroke model is a rather complex model to test the properties of a newly developed marker due to the BBB. We therefore decided to use a model in which compound delivery is easier for a first proof-of-concept., We implanted EL4 tumors into the flanks of mice and treated them with chemotherapy to induce apoptosis. We showed that prolonged circulation of XTEN-AnxA5 did indeed increase its accumulation in the tumors compared to wild-type AnxA5. Whether this will be sufficient to visualize dead or dying cells in the brain after experimental stroke remains to be elucidated in future studies. In addition to modifying AnxA5 itself, a continuous tracer infusion or step-down infusion may increase tracer delivery in comparison to single-bolus infusion.[40, 41]

In this thesis, I demonstrated that while fluorescently labeled AnxA5 for *in vivo* NIRF imaging specifically binds to dead or dying cells after focal cerebral ischemia in the mouse, a dual-labeled AnxA5 for SPECT is unsuitable for visualization of cell death. We developed a long-circulating version of AnxA5 with improved imaging properties that needs to be further evaluated in experimental stroke. Whereas AnxA5 seems to bind specifically to dead or dying cells, its suitability for brain imaging requires further elucidation using compounds designed to overcome the BBB and reach their respective targets.

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Affidavit

I, Marietta Zille certify under penalty of perjury by my own signature that I have submitted the thesis on the topic „Visualization of Cell Death after Experimental Stroke in the Mouse“. 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.

Declaration of any eventual publications

Marietta Zille had the following share in the following publications:

Publication 1: Bahmani P, Schellenberger E, Klohs J, Steinbrink J, Cordell R, **Zille M**, Muller J, Harhausen D, Hofstra L, Reutelingsperger C, Farr TD, Dirnagl U, Wunder A. Visualization of cell death in mice with focal cerebral ischemia using fluorescent annexin A5, propidium iodide, and TUNEL staining. J Cereb Blood Flow Metab. 2011
IF: **5.01 (2011)**
Contribution in detail: **30%**. Performed NIRF imaging experiments, partly drafted the manuscript and made critical revision of the manuscript and correspondence to reviewer comments.

Publication 2: **Zille M**, Harhausen H, De Saint-Hubert M, Michel R, Reutelingsperger CP, Dirnagl U, Wunder A. A dual-labeled Annexin A5 is not suited for SPECT imaging of brain cell death in experimental murine stroke. J Cereb Blood Flow Metab. 2014
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Contribution in detail: **20%**. Performed NIRF imaging experiments, partly drafted the manuscript.

Printouts of the Publications

Publication 1: Bahmani P, Schellenberger E, Klohs J, Steinbrink J, Cordell R, Zille M, Muller J, Harhausen D, Hofstra L, Reutelingsperger C, Farr TD, Dirnagl U, Wunder A. Visualization of cell death in mice with focal cerebral ischemia using fluorescent annexin A5, propidium iodide, and TUNEL staining. Journal of Cerebral Blood Flow & Metabolism (2011) 31, 1311–1320. http://dx.doi.org/10.1038/jcbfm.2010.233

Publication 2: Zille M, Harhausen H, De Saint-Hubert M, Michel R, Reutelingsperger CP, Dirnagl U, Wunder A. A dual-labeled Annexin A5 is not suited for SPECT imaging of brain cell death in experimental murine stroke. Journal of Cerebral Blood Flow & Metabolism 34, 1568-1570 (September 2014). http://dx.doi.org/10.1038/jcbfm.2014.115

Publication 3: Haeckel A, Appler F, Figge L, Kratz H, Lukas M, Michel R, Schnorr J, Zille M, Hamm B, Schellenberger E. XTEN-annexin A5: XTEN allows complete expression of long-circulating protein-based imaging probes as recombinant alternative to PEGylation. J Nucl Med March 1, 2014 vol. 55 no. 3 508-514. http://dx.doi.org/10.2967/jnumed.113.128108

Curriculum Vitae

Mein Lebenslauf wird aus datenschutzrechtlichen Gründen in der elektronischen Version meiner Arbeit nicht veröffentlicht.
My CV will not be published in the electronic version of my PhD thesis due to data privacy.

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Publications

Terzi MY, Casalis P, Lang V, **Zille M**, Bruendl E, Stoerr EM, Brawanski A, Vajkoczy P, Thomale U, Piña AP (2014) Effects of Pigment Epithelium Derived Factor on Traumatic Brain Injury (Restorative Neurology and Neuroscience, in revision)

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Reviews

Zille M, Farr TD, Przesdzing I, Muller J, Sommer C, Dirnagl U, Wunder A (2012) Visualizing cell death in experimental focal cerebral ischemia: promises, problems, and perspectives. J Cereb Blood Flow Metab 32:213-31

Contributions to Conferences

Keystone Symposium “The Chemistry and Biology of Cell Death”. February 2014. Santa Fe, USA. Contribution: In Vivo SPECT Imaging of Cell Death in Experimental Murine Stroke with 99mTc-labelled Annexin A5: A dead end? (poster)

Berlin Brain Days 2013. November 2013. Berlin, Germany. Contribution: In Vivo SPECT Imaging of Cell Death in Experimental Murine Stroke with 99mTc-labelled Annexin A5: A dead end? (talk)

XXVIth International Symposium on Cerebral Blood Flow, Metabolism and Function. May 2013. Shanghai, China. Contribution: Influence of pigment epithelium-derived factor on neurogenesis and angiogenesis after cerebral ischemia in the mouse (poster)

SfN Meeting Neuroscience 2012. October 2012. New Orleans, USA. Contribution: Specific imaging of cell death in experimental stroke using annexin A5 (poster)

7th International Symposium on Neuroprotection and Neurorepair. May 2012. Potsdam, Germany. Contribution: Specific imaging of cell death in experimental stroke using fluorescent and radioactively labeled annexin A5 (poster)

25. Treffpunkt Medizintechnik. May 2011. Berlin, Germany. Contribution: Specific imaging of cell death in experimental stroke using fluorescent annexin A5 (poster)

Neuronus 2011. April 2011. Krakow, Poland. Contribution: Characterization of cell death in primary neuronal and glial cells after Oxygen-Glucose-Deprivation (OGD) by flow cytometry (talk)

Molekulare Bildgebung 2010. November 2010. Seeon, Germany. Contribution: Specific Imaging of Cell Death in a Mouse Model of Stroke Using Fluorescent Annexin V (talk)

Berlin Brain Days 2010. November 2010, Berlin, Germany, Contribution: Characterization of cell death in primary neuronal and glial cells after Oxygen-Glucose-Deprivation (OGD) by flow cytometry (poster)

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