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DISSERTATION

Near-infrared fluorescence imaging of inflammation in stroke

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Abstract

Brain inflammation is a hallmark of cerebral ischemia, where it has been implicated in tissue damage as well as in repair. Imaging technologies that specifically visualize these processes are highly desirable. In this PhD thesis, the feasibility of planar near-infrared fluorescence (NIRF) imaging to visualize inflammation in a mouse model of focal cerebral ischemia (middle cerebral artery occlusion, MCAO) was explored. First, the feasibility of NIRF to visualize fluorochromes in the brain of mice was evaluated using an in-vivo phantom. Secondly, the use of NIRF-labeled bovine serum albumin (NIRF-BSA) for the detection of blood-brain barrier (BBB) permeability after MCAO was tested. Thirdly, it was investigated whether the inflammatory receptor CD40 can be specifically visualized in mice after MCAO using a fluorescent monoclonal antibody.

For preparing the in-vivo phantom, capsules containing different amounts of Cy5.5-dextran were implanted into the brain of mice and fluorescence reflectance imaging (FRI) and transillumination fluorescence imaging (TFI) were conducted. The detection limit of FRI and TFI was found to be 10^{-12} mol. TFI yielded significantly higher target-to-background ratios (TBR) than FRI at 10^{-11} mol ($p<0.05$). When background fluorescence is increased by intravenous injection of Cy5.5-dextran, the capsule is no longer detectable when the blood concentration reaches 0.4×10^{-6} mol/l.

For NIRF imaging of BBB permeability, mice were subjected to MCAO and NIRF-BSA was injected together with Evans blue (EB) at 4, 8 or 12 h after reperfusion. FRI was performed 4 h after compound injection. Higher fluorescence intensities over the ischemic hemisphere compared with the contralateral side were detected in mice at 8 and 16 h after reperfusion. No differences between hemispheres were seen in mice at 12 h after reperfusion. A good correspondence between the detection of high fluorescence intensities over the ischemic hemisphere and EB extravasation detected in brain slices was observed.

For NIRF imaging of the CD40 receptor, wild type and CD40-deficient mice were subjected to MCAO and were either intravenously injected with Cy5.5-CD40MAb or control Cy5.5-IgGMAb 80 h after reperfusion. FRI was performed 16 h after injection of the compounds. Wild type MCAO mice that received Cy5.5-CD40MAb showed significantly higher TBR compared to CD40-deficient MCAO mice injected with Cy5.5-CD40MAb and wild type mice injected with Cy5.5-IgGMAb ($p<0.001$). Confocal microscopy demonstrated vascular and parenchymal cellular distribution of the injected Cy5.5-CD40MAb in the ischemic region, with partial co-localization with activated microglia and blood-derived cells.

It was demonstrated that planar NIRF imaging can non-invasively detect fluorochromes in the brain of mice with high sensitivity. Using the technique, it was demonstrated that BBB permeability and the inflammatory receptor CD40 receptor can be specifically detected after cerebral ischemia.

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List of abbreviations

BBB	Blood-brain barrier
BSA	Bovine serum albumin
CCD	Charge-coupled device
CFDA	6-carboxylfluorescein diacetate
Cy5.5	CyDye 5.5
Cy5.5-CD40MAb	Anti-CD40 monoclonal antibody labeled with Cy5.5
Cy5.5-IgGMAb	IgG monoclonal antibody labeled with Cy5.5
EB	Evans blue
FITC	Fluoresceinisothiocyanat
FRI	Fluorescence reflectance imaging
FMT	Fluorescence-mediated tomography
FWHM	Full width half maximum
Iba-1	Ionized calcium-binding adapter molecule 1
ICAM-1	Intercellular adhesion molecule 1
IL-1	Interleukin-1
MAb	Monoclonal antibody
MCA	Middle cerebral artery
MCAO	Middle cerebral artery occlusion
MMPs	Matrix metalloproteinases
MRI	Magnetic resonance imaging
MW	Molecular weight
NHS	N-hydroxysuccinimide
NIRF	Near-infrared fluorescence
NIRF-BSA	NIRF-dye labeled BSA
OD	Optical density
PBS	Phosphate buffered saline
ROI	Region of interest
rtPA	Recombinant tissue plasminogen activator
SPIM	Single plane illumination microscopy
TBR	Target-to-background ratio
TFI	Transillumination fluorescence imaging
TNF-α	Tumor necrosis factor alpha
TRITC	Tetramethyl rhodamine isothiocyanate
TTC	Triphenyltetrazolium chloride

1. Introduction

Cerebral ischemia (stroke) is the result of the transient or permanent occlusion of a cerebral artery by an embolus or local thrombosis. Brain injury develops from a complex and dynamic series of pathophysiological events including excitotoxicity, peri-infarct depolarizations, inflammation and cell death [Dirnagl 1999]. At present, thrombolysis with recombinant tissue plasminogen activator (rtPA) is the only approved effective therapy when given within the first 3 hours after onset of cerebral ischemia [Broderick and Hacke, 2002]. Since the therapeutic time window leaves the majority of patients ineligible for treatment with rtPA, a search for novel therapeutic strategies has spurred.

Inflammation, which occurs with a delay of hours to days after the onset of the focal perfusion deficit, presents a promising therapeutic target which has the potential to expand the time window for therapy and thus the fraction of patients which might benefit from specific therapy [Dirnagl 2004]. While inflammation is considered to partake in the expansion of the ischemic lesion, certain aspects of inflammation have also been implicated in repair and recovery [Barone 1999, Manoonkitiwongsa 2001]. Therefore, therapeutic targeting of inflammation will have to rely on tools to diagnose the specific stage of inflammation in the individual patient.

In the past few years, imaging technologies using specific probes have emerged that enable the in-vivo imaging of physiological, metabolic and molecular function [Weissleder 2001, Massoud 2003]. Such technologies would provide powerful tools to further evaluate the role of inflammation after cerebral ischemia in experimental models and patients. Moreover, these technologies might be valuable in diagnosing inflammation (stage and severity), developing specific therapies, stratification towards specific therapies, and monitoring the response to therapeutic intervention.

At the molecular level, inflammation involves a complex cascade of mediators. Pro-inflammatory chemokines and cytokines (TNF-α, IL-1) are synthesized [Liu 1994], vascular adhesion molecules (e.g. ICAM-1) are upregulated [Wang 1994] and immune cells (e.g. neutrophils, monocytes) migrate from the blood to the brain parenchyma [Matsuo 1994, Akopov 1996]. Matrix metalloproteinases (MMPs) are upregulated [Justicia 2003,]. Microglia migrate to the site of injury and become activated [Rupalla 1998].

A process that could be studied with imaging is the impairment of the blood brain barrier (BBB). Inflammation after cerebral ischemia causes variable impairment of the BBB [Kuroiwa 1985, Nagaraja 2008].   The impairment of the BBB is mediated by MMPs and is thought to partake in edema formation, extravasation of plasma constituents and hemorrhagic transformation [Wang 2004]. Visualizing changes in the permeability of the BBB with imaging

would be valuable to study the pattern of extravasation and the contribution of this process to the expansion of the lesion and hemorrhagic transformation.

For a more inflammation-specific imaging approach, the CD40 receptor might be a suitable molecular target, because it is considered pro-inflammatory, to contribute to the expansion of the lesion, and is robustly up-regulated on various inflammatory cells in the brain. CD40 and its ligand CD 154 (CD40L) have been implicated in the regulation of the inflammatory response. The CD40 receptor is expressed on the surface of immune cells including B-cells and monocytes [Chen 2006]. In the central nervous system, CD40 is markedly upregulated after inflammatory stimulation on a variety of cells including microglia, astrocytes, and vascular endothelial cells [Chen 2006, Vowinkel 2006]. The interaction between CD40 and its ligand triggers a series of inflammatory cytokines and chemokines and induces the expression of vascular adhesion molecules [Omari 2004]. In experimental studies, targeted deletion of the CD40 receptor or its ligand CD154 has led to a reduction in infarct size [Ishikawa 2005].

A number of near-infrared fluorescence (NIRF) imaging devices with different setups, modes of data collection, and features have become available and have been tested for small animal imaging purposes in the last years [Weissleder 2003, Bremer 2003, Ntziachristos 2005, Licha 2005, Ntziachristos 2006]. NIRF imaging offers several advantages including high sensitivity, the use of non-ionizing radiation, as well as its demand for relatively simple and inexpensive instrumentation. Furthermore, the distribution of injected fluorescent probes can be evaluated with high spatial resolution using fluorescence microscopy. The vast majority of NIRF imaging systems are planar NIRF systems (reflectance fluorescence imaging, RFI, and transillumination fluorescence imaging, TFI). Fluorescence-mediated tomography (FMT) which allow the three-dimensional reconstructions of fluorochrome distribution with quantitative analysis have been reported [Ntziachristos 2002; Gurfinkel 2003, Ntziachristos 2005].

When planar NIRF imaging is applied to study inflammation in mouse models of cerebral ischemia one faces various methodological challenges. The fluorescent probe accumulated at the target, is embedded deep in tissues of different optical properties. This might affect the detection limit of the method and its sensitivity to background fluorescence. In addition, the administration of targeted fluorescent compounds can result in unspecific distribution of the compound due to an impaired BBB.

2. Aims

The aim of this PhD thesis was to investigate the feasibility of detecting stroke-induced inflammation with non-invasive planar NIRF imaging in a mouse model of focal cerebral ischemia:

First, the feasibility of non-invasive planar NIRF imaging to visualize fluorochromes in the brain of live pigmented mice was evaluated. For this purpose, an in-vivo phantom was developed where the presence of an accumulating fluorescent probe at a site of a lesion is simulated. The detection limit and the sensitivity to background fluorescence of planar NIRF were evaluated with the in-vivo phantom.

Secondly, the use of NIRF-labeled bovine serum albumin as a non-specific probe for the detection of BBB permeability with NIRF in a mouse model of focal cerebral ischemia was tested. NIRF-BSA was compared to Evans blue, a standard marker of plasma extravasation.

Thirdly, it was tested whether a NIRF-labeled monoclonal antibody can be used for the specific imaging of the inflammatory receptor CD40 in a mouse model of focal cerebral ischemia. To test the specificity of the imaging approach in-vivo, a NIRF-labeled IgG antibody without binding affinity to CD40 and CD40-deficient mice were used as controls. In addition, the distribution of the antibodies at the cellular level using microscopic techniques and immunohistochemistry was evaluated.

3. Materials and Methods

Animals

All experimental procedures conformed to institutional guidelines and were approved by an official committee (G0229/05, Lageso, Berlin, Germany). Seventy-two male C57Bl6/N mice (Bundesinstitut fuer Risikoforschung, Berlin, Germany) and four male B6.129P2-Cd40^{tm1Kik}/J mice (CD40-/-, Institute of Microbiology, ETH Zurich, Switzerland) weighing 18-26 g were housed under standard conditions.

In-vivo phantom

Capsules (volume of 0.106 µl) containing varying concentrations of Cy5.5-dextran solution were prepared and deposited into the left brain hemisphere of anesthetized mice (corresponding to 10^{-11} (n=13), 10^{-12} (n=4) or 10^{-13} (n=4) mol Cy5.5). In a subset of animals (10^{-11} mol Cy5.5, n=7), boluses of 0.2, 0.2, 0.4, 1.2, 1.6, 1.6 x 10^{-9} mol Cy5.5-dextran were subsequently injected into the tail vein (to give a blood concentration of 0, 0.1, 0.2, 0.4, 1, 1.8, and 2.6 x 10^{-6} mol/l, assuming a blood volume of 2 ml).

Focal cerebral ischemia

Middle cerebral artery occlusion (MCAO) was performed as described [Laufs 2000]. Briefly, a monofilament was introduced into the common carotid artery under isoflurane anesthesia, advanced to the origin of the middle cerebral artery (MCA), and left there for 60 minutes, until reperfusion. Sham-operation involved surgical procedures, without occlusion of the MCA.

Blood-brain barrier permeability imaging protocol

Indotricarbocyanine BSA conjugate (NIRF-BSA, 50 mg/kg, 3.5 dye molecules per protein on average) and Evans blue (EB, 50 mg/kg) were injected intravenously in MCAO mice 4, 8 and 12 h after reperfusion (n=4, n=5 and n=5 respectively). The conjugate was allowed to circulate for 4 h before NIRF imaging. Non-invasive NIRF imaging was performed under chloralhydrate anesthesia. For ex-vivo imaging, brains were removed from the skull and coronal brain slices of 1 mm thickness were cut in a brain matrix using a razor blade. Dye concentration in brain slices was estimated by using a reference solution of similar bulk optical properties (20 % milk, 80 % water) containing 10^{-6} mol/l of the NIRF dye. Brain slices were digitized for evaluation of EB extravasation.

CD40 imaging protocol

Fluorescently labeled antibodies were provided by Prof. Dr. Kristof Graf and PD Dr. Michael Gräfe (German Heart Center Berlin). Rat anti-CD40 monoclonal antibodies (Cy5.5-CD40MAb, clone FGK45) and rat IgG2A monoclonal antibodies (Cy5.5-IgGMAb, clone 1d10)

were labeled with Cy5.5-NHS ester at an average Cy5.5-antibody-ratio of 3:1. Twenty-two wild type mice either received Cy5.5-CD40MAb (MCAO: n=11; Sham: n=5), or Cy5.5-IgGMAb (MCAO: n=7) as a control for unspecific distribution. Four CD40-deficient MCAO mice were injected with Cy5.5-CD40MAb. Non-invasive and ex-vivo NIRF imaging was performed 16 h after injection of the compounds (i.e. 96 h after reperfusion). Coronal brain sections were prepared for lesion analysis and immunohistochemistry.

To study probe distribution, six wild type MCAO mice were either injected with Cy5.5-CD40MAb or Cy5.5-IgGMAb (n=3 each) 80 h after reperfusion. Single plane illumination microscopy (SPIM) was performed 16 h after injection of the labeled compounds.

To give an approximation on the overall contribution of blood-derived cells to the CD40-NIRF signal, wild type MCAO and sham-operated mice (n=2 each) were injected with Cy5.5-CD40MAb 80 h after reperfusion and received an intrasplenic injection of CFDA into the spleen as described [Bechmann 2005]. As controls, MCAO mice were intravenously injected with CFDA or received an intrasplenic injection of PBS (n=2 each). All brains were taken for confocal microscopy 16 h after injection of the CFDA.

Magnetic resonance imaging

Magnetic resonance imaging (MRI) was performed on a 7 T animal scanner with a 16 cm horizontal bore magnet and a shielded gradient with a maximum strength of 300 mT/m. A T1-weighted 2D turbo spin-echo sequence was used (TR 1000 ms, TE_{eff} 13.2 ms, 4 averages, total scan time 8 min 30 sec).

Near infrared fluorescence imaging

Intensity-controlled cooled laser diodes emitting at 682 nm (30 mW) and 780 nm (7 µW) were coupled to an optical switch. The switch allowed to direct the laser light either to the throat of the animals (transillumination fluorescence imaging, TFI) or to illuminate the head of the mice from above with two overlapping light cones (fluorescence reflectance imaging, FRI). Cy5.5 and NIRF dye were excited at 682 nm. Fluorescence emission was collected with a CCD camera fitted above the head of the animal. The CCD camera was equipped with appropriate filter sets (Cy5.5: three 710 nm interference filters with FWHM 10 nm; NIRF dye: two 780 nm interference filters FWHM 10 nm and a RG filter). Data acquisition times ranged from 1 to 60 s, depending on the intensity of the fluorescence signal. To correct for illumination inhomogeneities and to provide an anatomical reference, reference images were taken at 682 nm for Cy5.5 (OD4 filter) or for NIRF dye at 780 nm (two interference filter 780 nm).

Data processing and image analysis

NIRF images were normalized and corrected for illumination inhomogeneities as described [Ntziachristos 2005II]. Region of interest (ROI) were selected over the right and left hemisphere. The average fluorescence intensity of all pixels within the ROI was calculated. Target-to-background ratios (TBR) were calculated by dividing the average fluorescence intensity of the ROI over the left hemisphere by those of the right hemisphere.

Lesion determination

Brain slices were incubated in a 2 % TTC solution at 37 °C for 30 minutes. An image of each slice was analyzed with NIH Image software. Lesion volumes were calculated as described [Laufs 2000].

Immunohistochemistry and confocal microscopy

Immunohistochemistry and confocal microscopy were performed in collaboration with Dr. Golo Kronenberg and Prof. Dr. Matthias Endres (Experimental Neurology, Charité Universitätsmedizin Berlin). Cryostat sections were cut between 1.6 to -1.3 from bregma and were stained with TRITC-labeled anti-Iba1 antibodies. The cortex, striatum and peri-ischemic territory of three coronal sections were inspected for each brain. To estimate the number of Iba1 and CFDA positive cells that are Cy5.5 fluorescent, fifty Iba1 and fifty CFDA positive cells in each section in randomly selected high power fields were examined respectively.

Single plane illumination microscopy

SPIM was performed in collaboration with Dr. Klaus Greger and Dr. Ernst Stelzer (European Molecular Imaging Laboratory, Heidelberg) as described [Huisken 2004].

Statistical analysis

Comparisons between TBR were made using an unpaired Students t-test or a 1-way ANOVA followed by Bonferroni post-test, were appropriate. TBR were plotted against lesion volumes, followed by a linear regression analysis to calculate R^2 and to determine the regression equation.

4. Results

The results of this PhD thesis have been included in publications as shown in the publication list in the appendix.

Non-invasive near-infrared imaging of fluorochromes in in-vivo phantoms [publ. 1]

Capsules were positioned with high reproducibility as determined by MRI. The deviation of the intended implantation depth and distance from the midline was below 0.5 mm.

Fluorescent capsules containing the lowest amount of dye (10^{-13} mol) could neither be detected with FRI nor with TFI. The capsules containing 10^{-12} and 10^{-11} mol NIRF dye, could be clearly identified with FRI and TFI. TBR calculated from FRI images of mice with capsules containing 10^{-13} mol were significantly different from the corresponding FRI values of mice with capsules containing 10^{-12} mol and 10^{-11} mol (approximately 1, 2 and 3 respectively, $p<0.05$). TBR of TFI images were significantly higher than FRI only in mice which contained capsules of 10^{-11} mol (4 versus 3, $p<0.05$).

At a blood dye concentration of 0.4×10^{-6} mol/l, the capsule containing 10^{-11} mol Cy5.5 can still be detected, but is no longer detectable when the dye concentration in blood is increased to 1×10^{-6} mol/l. TBR calculated from TFI images decreased with increasing dye concentrations, approximating 1 when the capsules is no longer detected.

Non-invasive near-infrared fluorescence imaging of blood-brain barrier permeability after cerebral ischemia [publ. 2]

Higher fluorescence intensities over the ischemic hemisphere compared with the contralateral side (TBR≥1.1) were detected with FRI in three out of four mice 8 h after reperfusion and in two out of five animals 16 h after reperfusion. No differences between hemispheres (TBR<1.1) were seen with FRI in one out of four animals 8 h after reperfusion, all five mice 12 h after reperfusion and 3 out of five animals 16 h after reperfusion. NIRF imaging of the brain after removal from the skull confirmed intense fluorescence over the ischemic hemisphere in those mice that had shown TBR≥1.1. In mice that showed TBR<1.1 the fluorescence was found to be evenly distributed over both hemispheres. Only in one mouse fluorescence over the ischemic territory was revealed with ex-vivo NIRF where no fluorescence had been detected non-invasively 16 h after reperfusion. From NIRF images of coronal brain slices the concentration of NIRF-BSA that accumulates in the ischemic lesion was calculated to be about 10^{-6} mol/l.

EB extravasation was macroscopically detected in brain slices as a diffuse blue tissue coloration of various intensities. EB extravasation was detected in three out of four mice at 8 h after reperfusion and two out of five mice 16 h after reperfusion. No EB extravasation was

observed in mice at 12 h after reperfusion. A good correspondence of EB extravasation detected ex-vivo with fluorescence observed in the ischemic area on FRI images was observed.

In-vivo imaging of the inflammatory receptor CD40 after cerebral ischemia [publ. 3]

No differences between hemispheres were seen with FRI in sham-operated mice that received Cy5.5-CD40MAb, MCAO animals that received Cy5.5-IgGMAb, and CD40-/- MCAO mice that received Cy5.5-CD40MAb. Higher fluorescence intensities over the ischemic hemisphere compared with the contralateral side were detected non-invasively only in wild type MCAO mice injected with Cy5.5-CD40MAb. TBR were statistically significant lower in sham operated mice that received Cy5.5-CD40MAb, MCAO animals that received Cy5.5-IgGMAb, and CD40-/- MCAO mice that received Cy5.5-CD40MAb compared to MCAO mice that received Cy5.5-CD40MAb (0.94±0.08, 1.0±0.14 and 0.95±0.08 vs. 1.47±0.16 respectively, p<0.001).

The corresponding ex-vivo NIRF images showed equally low fluorescence intensities over both hemispheres in brains of sham-operated animals that were injected with Cy5.5-CD40MAb. A low signal over the ischemic hemisphere was seen in MCAO animals injected with Cy5.5-IgGMAb, and CD40-/- mice that received Cy5.5-CD40MAb. Intense fluorescence was only seen over the ischemic hemisphere of MCAO mice that had received Cy5.5-CD40MAb.

A lesion was clearly delineated by TTC-staining in all MCAO animals 96 h after reperfusion. CD40-/- and wild type MCAO mice showed equal lesion size. In sham-operated animals receiving Cy5.5-CD40MAb, weak fluorescence was evenly distributed over the brain slice. Areas of low fluorescence intensities, corresponding to the pallor in TTC-staining, were seen in MCAO animals injected with Cy5.5-IgGMAb and the CD40-/- mice that received Cy5.5-CD40MAb. Areas with high fluorescence intensities were observed over the ischemic region in MCAO mice that had received Cy5.5-CD40MAb. Linear regression analysis demonstrated no linear correlation between the calculated TBR and lesion volume with a $R^2=0.159$.

Inspection with SPIM revealed higher fluorescence intensities in the ischemic territory in mice injected with Cy5.5-CD40MAb compared to mice that received Cy5.5-IgGMAb. The signal in both animal groups was found to be associated with vessels. In mice that received Cy5.5-CD40MAb additional signal in the form of dots of cellular size was detected, whereas no dots were visible in mice injected with Cy5.5-IgGMAb. In the parenchyma of those mice a scarce and diffusely distributed fluorescence was observed.

Confocal microscopy demonstrated a large number of Iba1 positive cells of similar density in the ischemic cortex of animals that had received Cy5.5-CD40MAb and Cy5.5-IgGMAb. Mice

that received Cy5.5-CD40MAb showed a partial co-localization of fluorescence from the injected compound with the Iba1-staining. Confocal analysis revealed co-localization with CD40MAb in about half of the Iba1 positive cells. By contrast, fluorescence from control IgG was less abundant and was seen as a diffuse signal, without cellular localization.

Confocal microscopy of mice that received an intrasplenic injection of CFDA in addition to Cy5.5-CD40MAb administration showed fluorescence from CFDA positive cells in the ischemic lesion, which was partially co-localized with the fluorescence from Cy5.5-CD40MAb. Confocal analysis of CFDA positive cells showed a co-localization with Cy5.5-CD40MAb in every second cell. In the three control groups, no CFDA positive cells were observed in the brain parenchyma.

5. Discussion

Planar NIRF imaging using fluorescent probes is a promising technology for visualizing biological events non-invasively in small animals. In summary, in this PhD thesis it was demonstrated that planar NIRF imaging can non-invasively detect fluorochromes in the brain of mice with high sensitivity and that the technique can be used to detect BBB permeability and the inflammatory receptor CD40 after cerebral ischemia.

When planar NIRF is applied to study inflammation in mouse models of cerebral ischemia several technical issues have to be considered. NIRF is highly sensitive in detecting small amounts of fluorescent probes in small animals [Schulz 2004, Graves 2005]. When a compound accumulates at targets in the brain, it is embedded deep in tissues of different optical properties. The ability to detect the probe in the brain non-invasively with planar NIRF will depend on the amount of probe accumulated at the target, the depth, and the presence of background fluorescence in the overlaying tissues. Using an in-vivo phantom it was shown that an amount of 10^{-12} mol of the NIRF fluorochrome Cy5.5 can be detected in the brains of live pigmented mice when no background fluorescence is present. The presence of relatively low background fluorescence (dye concentration in blood of 1×10^{-6} mol/l) can extinguish the signal when the amount of fluorochrome in the capsule is high (10^{-11} mol).

It was recently reported that TFI show higher TBR than RFI [Ntziachritos 2005II]. Using the in-vivo phantom, significantly higher TBR were calculated from TFI compared to FRI at capsules containing 10^{-11} mol Cy5.5. This might be explained by the fact that in RFI the excitation photons are directly reflected on the skin towards the detector, while in TFI the number of reflected photons is reduced because the excitation photons travel through the absorbing tissue of the brain and skull. In addition, autofluorescence by excitation of endogenous chromophores in the skin is reduced when applying TFI. While this observation favors TFI as mode of data acquisition for imaging the brain, the application of TFI in MCAO and sham-operated mice has been found to be not feasible. The scar that results from surgery prevents a good coupling of the fiber to the throat of the animals.

The use of TBR has been established using the in-vivo phantom. TBR were used to make comparisons between mice in BBB permeability and CD40 receptor imaging. A limitation of planar NIRF is that it does not allow for absolute quantification. However, quantification has been reported for fluorescence-mediated tomography [Ntziachristos 2002, Ntziachristos 2006].

A first application of planar NIRF was to visualize the permeability of BBB using NIRF-BSA as a non-specific probe. The NIRF-fluorescent probe has been compared to EB. EB binds to albumin in the blood plasma when administered intravenously, and is used as a standard marker for the ex-vivo assessment of BBB impairment [Kuroiwa 1984, Uyama 1988, Belayev

1996]. Variable permeability of the BBB has been demonstrated with EB. In cats that underwent 1 h MCAO, EB extravasation was observed shortly after reperfusion and at 5 and 72 h after reperfusion [Kuroiwa 1984]. In rats that were subjected to 2 h MCAO, EB leakage was found 5 and 50 h after reperfusion [Belayev 1996]. Different patterns were also observed when using radiolabeled tracers of lower molecular [Preston 2002].

In this study, higher fluorescence intensities over the ischemic hemisphere were detected with RFI 8 and 16 h after reperfusion. Fluorescence showed a variable degree within the groups. No fluorescence was observed 12 h after reperfusion. EB extravasation and fluorescence detected with FRI showed a good correspondence. Only in one mouse EB extravasation was seen, but not differences in fluorescence intensities between the ischemic and contralateral hemisphere were observed with FRI. In this mouse wounds in the skin overlaying the non-ischemic hemisphere of this mouse were seen which could have caused an extravasation of NIRF-BSA to the skin precluding the observation of differences in fluorescence intensities.

Taken together, this suggests that NIRF-BSA might be used as a non-invasive equivalent to EB for the assessment of BBB permeability.

A step towards more specific imaging of inflammation was to test the use of a fluorescent antibody as a targeted probe against the CD40 receptor. The use of a targeted probe requires in-vivo testing for specificity of the signal, because the impairment BBB might lead to a parenchymal distribution of the compound unrelated to specific binding. To test the specificity, Cy5.5-IgGMAb, which has no binding affinity for CD40, was injected in wild type MCAO mice and CD40-deficient MCAO mice were injected with Cy5.5-CD40MAb as control. In these mice no differences between hemispheres were seen on FRI images. When the brains were removed from the skull and NIRF imaged, fluorescence intensities in the ischemic tissue were slightly higher compared to non-ischemic tissue. The amounts of fluorescent compounds in the ischemic tissue were clearly below the detection limit and were hence not detected with FRI.

Inspection of tissue samples with SPIM revealed marked differences in the distribution of Cy5.5-CD40MAb and control Cy5.5-IgGMAb. In the ischemic tissue of wild type mice that received the CD40-antibody highly fluorescent dots of cellular size were detected in the parenchyma and associated with vessels. In contrast, the signal was rather diffuse and scarcely visible in mice that received fluorescent IgG. Taken together, this gives raise to the notion that the signal after injection of fluorescent-labeled CD40 antibody is largely due to specific binding and/or uptake of the compound rather than due to leakage.

Using immunohistochemistry and confocal microscopy it was found that fluorescence from the injected CD40-antibody was largely associated with Iba1-staining (around 50%). The

fluorescence not associated with Iba1-staining might represent cross-sectioned capillaries filled with circulating compound, because mice were not perfused to prevent wash-out of the probe, or other cell types that express CD40, such as activated endothelial cells or B-cells. Cell tracking experiments with CFDA revealed the presence of CFDA positive cells in the ischemic territory of MCAO mice which showed partial co-localization with the fluorescence from injected Cy5.5-CD40MAb. Cells infiltrating the ischemic brain tissue could either have carried the fluorescently labeled CD40-antibody inside the brain, or have bound and/or taken up the label within the brain parenchyma. In summary, the CD40-targeted antibody is mainly found attached to cell populations (mainly microglia, monocytes) involved in stroke-induced brain inflammation, with a substantial contribution of blood-derived cells.

Ninety-six hours after reperfusion was chosen as an experimental paradigm for the subacute stage of inflammation. It was recently demonstrated that targeted deletions of CD40-receptor or CD154 lead to a significant reduction of infarct sizes 24h after MCAO [Ishikawa 2005]. In this study, no differences between the lesion size of CD40-/- and wild type mice were seen. A correlation between lesion volume and TBR of wild type MCAO mice that received Cy5.5-CD40-MAb were not observed. This could suggest that a correlation between CD40 and lesion size is likely to be time dependent.

While NIRF imaging of small animals offers several advantages, its application in clinical settings is more challenging [Ntziachritos 2006, Vogel 2006]. It has been demonstrated that depth-resolved in-vivo NIRF measurements can be performed after intravenous injection of indocyanine green even in the brain of human adults [Liebert 2006]. However, these approaches are limited by the ultimate penetration depth of the excitation and emission light which was found to be approximately 15 mm into the head, and thus a few millimeters into the brain. Since the amount of light needed to illuminate deeper structures in the brain is limited by the heat-load on the tissue, these applications are restricted to the monitoring of cortical structures [Steinbrink 2008]. Thus clinical imaging in humans requires imaging modalities that are not limited by depth penetration like MRI, PET or SPECT.

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Appendix

List of publications

Publication 1:

Klohs J, Steinbrink J, Nierhaus T, Bourayou R, Lindauer U, Bahmani P, Dirnagl U, Wunder A (2006) Non-invasive near-infrared imaging of fluorochromes within the brain of live mice: an in-vivo phantom study. Mol Imaging 5(3): 180-7

80%

Individual contribution: conceived and designed the research, established in-vivo phantom model, acquired the imaging data, analyzed and interpreted the data, performed statistical analysis, drafted the manuscript, corresponded to reviewer comments

Publication 2:

Steinbrink J, Villringer A, Liebert A, Wabnitz H, Macdonald R, Obrig H, Wunder A, Bourayou R, Betz T, **Klohs J**, Lindauer U, Dirnagl U, Villringer A (2008) Towards noninvasive molecular fluorescence imaging of the human brain. Neurodegen Dis 5(5): 296-303

40%

Individual contribution: performed in-vivo model of cerebral ischemia, acquired and analyzed imaging data, partially drafted the manuscript and made critical revision of the manuscript

Publication 3:

Klohs J, Gräfe M, Graf K, Steinbrink J, Dietrich T, Stibenz D, Bahmani P, Kronenberg G, Harms C, Endres M, Lindauer U, Greger K, Stelzer E, Dirnagl U, Wunder A (2008) In-vivo imaging of the inflammatory receptor CD40 after cerebral ischemia using a fluorescent antibody. Stroke 39(10): 2845-2852

80%

Individual contribution: conceived and designed the research, performed in-vivo model of cerbral ischemia, acquired imaging data, performed histology and infarct analysis, acquired single plane illumination microscopy data, analyzed and interpreted data, performed statistical analysis, drafted the manuscript, responded to reviewer comments

Berlin, May 14th 2008

Jan Klohs   Prof. Dr. med. Ulrich Dirnagl

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Eidesstattliche Erklärung

Ich, Jan Klohs, geboren am 03.04.1978 in Frankfurt/Oder, erkläre hiermit an Eides statt, dass ich die vorliegende Dissertationsschrift ohne unerlaubte Hilfe Dritter selbstständig angefertigt habe und die benutzte Literatur und Hilfsmittel vollständig angegeben sind. Die Dissertationsschrift stellt keine Kopie anderer Arbeiten dar und wurde an keiner anderen Fakultät eingereicht.

Berlin, den 14.05.2008

Jan Klohs

Curriculum vitae

Mein Lebenslauf wird aus Datenschutzgründen in der elektronischen Version meiner Arbeit nicht mit veröffentlicht.

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Original articles

Klohs J, Steinbrink J, Nierhaus T, Bourayou R, Lindauer U, Bahmani P, Dirnagl U, Wunder A (2006) Non-invasive near-infrared imaging of fluorochromes within the brain of live mice: an in-vivo phantom study. Mol Imaging 5(3): 180-7 [not graded yet]

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Publication 1:

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Publication 2:

Steinbrink J, Villringer A, Liebert A, Wabnitz H, Macdonald R, Obrig H, Wunder A, Bourayou R, Betz T, Klohs J, Lindauer U, Dirnagl U, Villringer A (2008) Towards noninvasive molecular fluorescence imaging of the human brain. Neurodegen Dis 5(5): 296-303

Publication 3:

Klohs J, Gräfe M, Graf K, Steinbrink J, Dietrich T, Stibenz D, Bahmani P, Kronenberg G, Harms C, Endres M, Lindauer U, Greger K, Stelzer E, Dirnagl U, Wunder A (2008) In-vivo imaging of the inflammatory receptor CD40 after cerebral ischemia using a fluorescent antibody. Stroke 39(10): 2845-2852