AN MRI CONTRAST AGENT TARGETED FOR ACTIVATED PLATELETS ALLOWS DETECTION OF THROMBOSIS AND THROMBOLYSIS IN AN IN VIVO MOUSE MODEL

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Introduction Upon rupture of vulnerable plaques, initial aggregation and activation of platelets plays a pivotal role[1], mediating further activation of the coagulation cascade. To allow *in vivo* imaging of activated platelets in wall adherent, non-occlusive arterial thrombosis, we applied a MRI contrast agent consisting of MPIOs and unique single-chain antibodies designed to selectively recognize ligand-induced binding sites (LIBS) of the activated GPIIb/IIIa-receptor[2] in a mouse model of ferric chloride induced non-occlusive carotid thrombosis. This contrast agent allowed identification of small platelet amounts in non-occlusive thrombosis with excellent MRI contrast properties. Furthermore, we were able to monitor thrombolysis and therefore decreasing local platelet activity by *in vivo* MRI after applying urokinase to a subgroup of animals, which is an important characteristic for future clinical applicability of such a platelets-based MRI contrast agent.

Methods The relaxivities r_1, r_2 and r_2^* where determined at 9.4 Tesla according to the literature[3] in order to estimate the MPIO-induced susceptibility properties. The monoclonal antibody (mAb) anti-LIBS 145 binds to GPIIb/IIIa only in its active conformation, and demonstrates strong binding to ADP-activated platelets in the presence of fibrinogen. Construction and binding of the LIBS-MPIO contrast agent to activated platelets as well as its functional evaluation has been described elsewhere [2]. Autofluorescent cobalt-functionalised MPIOs (1 µm) were conjugated to the histidine-tag of the LIBS/control single-chain antibody referring to the protocol of the manufacturer (Dynal Biotech, Oslo, Norway).

Care and use of laboratory animals followed the national guidelines and was approved by the institutional animal care and ethics committees of the University of Freiburg, Germany. 10-11 week old male C57BL/6 mice weighting 24+/-0,2 g (Charles River, Sulzbach, Germany) were used. Mice were anesthetized by i.p. injection with ketamine (100 mg/kg BW) and xylazine (5 mg/kg BW). To obtain a semi-occlusive thrombosis of the carotid artery, we adapted an established model[4] of ferric chloride induced carotid artery thrombosis. It is induced by applying a piece of filter paper (1x2 mm, GB003) saturated with ferric chloride (3-10 % solution) for 3 minutes under the right carotid artery, and occludes the vessel lumen in ranges of 15-45%. Thereafter a venous catheter connected with a 1 ml syringe containing 0.9% saline via a 1.2m long tube was placed in the tail vein. The animals were monitored regarding ECG and breathing-rate and body temperature was supported through a warm water tube integrated in the animal bed. During MRI anaesthesia was continuously switched from ketamin to 1-1.6% isoflurane in O_2 starting at a heart rate rising above 190 beats/min.

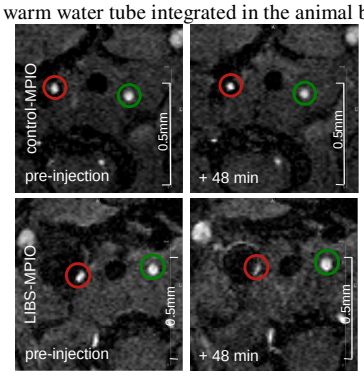

Fig. 1 Transversal slices of injured right (red), and non-injured left carotid artery (green).

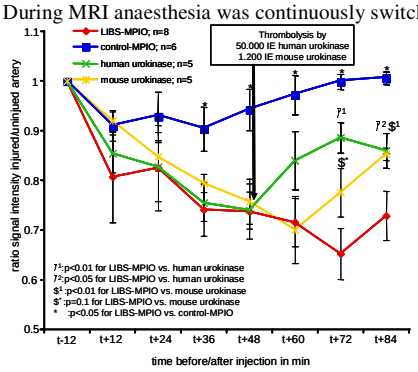

Fig. 2 Quantification of signal voids. LIBS-MPIO attenuation was highly significant after 48'.

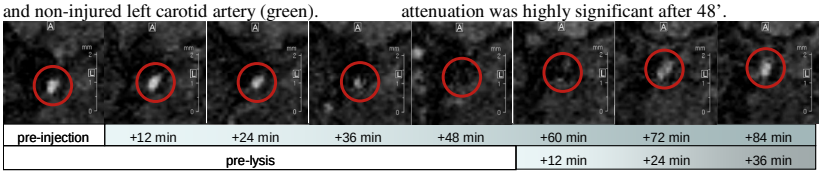

Fig. 3 Zoomed images of transversal slices of the injured right carotid artery demonstrate the signal drop after LIBS-MPIO injection and the reappearance of the vessel signal due to mouse urokinase.

MRI was done on a 94/20 Bruker BioSpec, Bruker, Germany system employing a quadrature whole body birdcage resonator (35mm inner diameter). Imaging consisted of a pilot scan, and a respiration gated coronal 2D gradient echo sequence orientated parallel to the trachea on which the 3D slab was planed. This slab included the bifurcation and the upstream carotid arteries beyond the lesion. The 3D FLASH sequence had a TE/TR of 2.8ms/20ms, a bandwidth of 55kHz, an echo position of 25%, a matrix of 256x256x96, a resolution of 105 x 105 x 78 µm, a t_{acq} of 12'17'' and an alpha of 15°. It was run once before and continuously for 84 min after contrast agent injection. The contrast agent was injected via the tail vein catheter with either the LIBS-MPIO or control-MPIO contrast agent (4 x 10^8 MPIOs) in a total volume of 200µl saline[5]. Two other groups of LIBS-MPIO-injected animals received thrombolysis by either human urokinase (50.000 IU) or the recombinant mouse urokinase[6] (1200 IU) in a total volume of 250 µl. For quantification 15 slices including the thrombosis where chosen 3-6 mm caudally of the bifurcation. A 3D ROI was defined over these slices selecting 5 image pixels centered within the artery in each slice once for the contra lateral and once for the lesion side. For each time point we calculated the quotient of mean signal intensity of the ROI in the thrombotic vessel divided by the mean of the ROI in the contra lateral vessel. These quotients where then normalized on the baseline 3D FLASH starting value.

Results Relaxivity measurements revealed a negligible r_1 of 0.05 +/-0.011 $mmol^{-1}s^{-1}$, an r_2 of 121.2 +/- 6.8 l $mmol^{-1}$ s^{-1} and an r_2^* of 494.4 +/- 25.0 l $mmol^{-1}$ s^{-1}(data not shown). After contrast agent injection, an initial signal drop occurred in both groups after 12 min. However, the visible signal decrease was stronger in LIBS-MPIO and was highly significant after 48 min ($p<0.01$, Fig. 2). We observed a significant signal increase in the animals injected with human urokinase after 24 min (n=5), and in animals with mouse urokinase after 36 min (n=5) compared to LIBS-MPIO.

Discussion An r_2^* of 494.4 $l\,mmol^{-1}s^{-1}$ leads to a concentration of 0.16 mMol iron required for a 20% signal attenuation using the 3D FLASH sequence with TE 2.8ms. Therefore one would expect ~46 MPIOs in the histological volume. Histology confirmed 20-40 MPIOs per slice. These slightly lower numbers can be explained through the non-physiological high shear-stress conditions generated through the flushing procedure before tissue harvesting.

The initial signal drop observed in the control group might be due to temporal non specific adhesion of MPIOs at the thrombus. The activation yield of murine plasminogen with human uPA is 4-fold lower than with mouse uPA[7]. Therefore more active units human uPA are needed to achieve a similar rate of thrombolysis when compared to mouse uPA. The human urokinase used in this study contains a mixture of non-activated scuPA and the activated form two-chain uPA. In contrast, the used mouse urokinase was a non-active proenzyme scuPA converted to the active two-chain form after injection. This difference explains the delayed thrombolysis between mouse and human urokinase.

Conclusion We were able to image wall adherent thrombosis using a contrast agent that specifically detects activated platelets. Futhermore, we directly monitored the success of thrombolytic treatment. LIBS-MPIO-induced signal void was a quantitative measure of MPIO-binding and thrombosis size, and rapid intravascular MPIO-clearance allowed imaging by high resolution *in vivo* MRI with excellent contrast properties. Thus, the described targeted MRI contrast agent represents a novel and unique non-invasive technique that allows detection and quantification of thrombi and can be used to monitor success or failure of thrombolytic therapy. These properties are a promising basis for further development and application of MPIO-based contrast agents for the potential detection of vulnerable, rupture-prone atherosclerotic plaques before they cause myocardial infarction or stroke.

Literature **1)** Weyrich A et al *Curr Pharm Des.* 2007;13(16):1685; **2)** Schwarz M et al. *J Pharmacol Exp Ther.* Mar 2004;308(3):1002; **3)** Rohrer M et al. *Invest Radiol.* Nov 2005;40(11):715; **4)** Westrick RJ et al. *Arterioscler Thromb Vasc Biol.* 2007;27(10):2079; **5)** McAteer MA et al. *Nat Med.* 2007 in press; **6)** Belin et al. *Eur J Biochem.* 1985;148(2):225; **7)** Lijnen HR et al. *Eur J Biochem.* 1996;241(3):840

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