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Quantitative Studies of the Internalization of Iron Oxide Nanoparticles by Mesenchymal Stem Cells

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Introduction

Nanoparticles of iron oxide have been recently used to magnetically label cells *in vitro*. Small particles of iron oxide (SPIO, hydrodynamic diameter: 80-150 nm) and ultra small particles of iron oxide (USPIO, hydrodynamic diameter: 20-30 nm) were used in this context. Bone marrow mesenchymal stem cells (MSCs) have been magnetically labelled for a MRI monitoring of their migration after implantation in the injured rat brain (1). Our aim is to quantify the internalization of SPIO by MSCs, to study the mechanisms of internalization of SPIO by MSCs, and to compare the internalization of SPIO and of USPIO by MSCs. While stem cells magnetic labeling can be realized with nanoparticles of iron oxide pre-incubated with a lipofection or transfection agent (2-3), in our case, the internalization have been performed with the native particles.

Methods

MSCs were isolated from 8 weeks old rats femurs. After deep anesthesia with Nembutal, rats were sacrificed and their femurs were surgically extracted. Whole bone marrow extract was plated in 6-wells tissue culture dishes and 24 hours later, non-adherent cells were removed from the culture by replacing the medium, in order to isolate MSCs (4). MSCs were cultured during 12 to 15 days before the incubation with SPIO or USPIO. Wells contained 4 ml of Dulbecco's modified eagle medium supplemented with 10% FBS and 1% antibiotic-antimycotic solution. MSCs were incubated with SPIO at 0, 25, 50, 100 and 200 µg of iron/ml, or with USPIO at 100 and 200 µg of iron/ml during 48 hours in order to evaluate the capture of the particles as a function of the iron concentration in the incubation medium and of the particle size. MSCs were also incubated with SPIO at 100 µg of iron/ml during 3, 6, 9, 12, 24 and 48 hours to estimate the kinetic of internalization of SPIO by MSCs. Internalization mechanisms were inhibited by pre-incubation of the cells during 4 hours with cytochalasin B (to prevent actin microfilaments polymerization) or colchicine (to prevent microtubules polymerization) (5). Internalization of SPIO and USPIO by MSCs was evaluated by T_2-weighted MRI (4.7 T) and colorimetry after iron staining with Prussian blue on mineralized cells. For MRI, cells were resuspended in PCR tubes at a concentration of $5x10^5$ cells/ml in 2% gelatin and the following acquisition sequence was used: (TR=2000ms, TE=20ms, 16 echoes, NA=2, matrix = 256 x 256). PARAVISION software was used to measure T_2 values on the MR images.

Results

After incubation with SPIO (increasing incubation concentrations and increasing incubation times) , R_2 values obtained from the T_2-weighted MR images and iron concentration follow the same trend (Fig. 1a & 1b). USPIO are less internalized than SPIO by MSCs (Fig. 1c), and both cytochalasin B and colchicin have an inhibitory effect on the internalization of SPIO by MSCs (Fig. 1d).

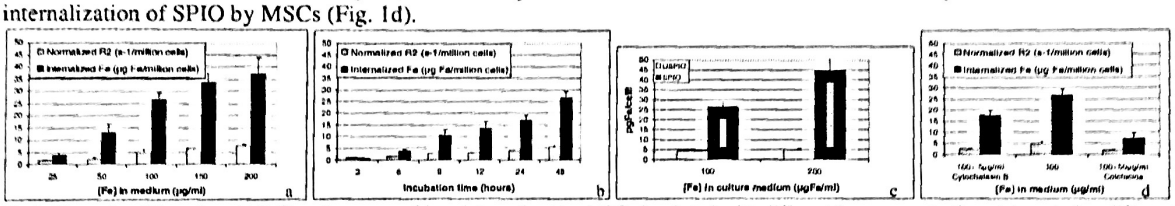

Fig.1. Normalized R_2 values (4.7 T) and amount of iron internalized by MSCs incubated with different iron concentrations (a) or during different times with 100 µg Fe/ml (b). Amount of iron internalized by MSCs incubated with 100 and 200 µgFe/ml SPIO or USPIO (c). Normalized R_2 values (4.7 T) and amount of iron internalized by MSCs incubated with 100 µgFe/ml, pre-incubated with cytochalasin B or colchicine (d).

Discussion and conclusions

MRI and colorimetry allow for the quantification of internalized iron. A decrease of the normalized R_2 value of cells incubated with 100 µgFe/ml is observed in the presence of colchicine and cytochalasin B, suggesting that at least two processes are involved in the endocytosis of SPIO by MSCs. The first onedepends on actin microfilaments polymerization, like macropinocytosis, and the other one depends on microtubules polymerization, like pinocytosis. Size also seems to be an important parameter for the capture of iron oxide nanoparticles by MSCs, since USPIO are less internalized than SPIO.

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Molecular Targeting of Angiogenic Blood Vessels in Atherosclerotic Plaques with a New Low Molecular Weight Non-Peptidic RGD Mimetic

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Introduction. Atherosclerosis represents the leading cause of death and illness in developed countries and will soon become the pre-eminent health problem worldwide. Recent evidence suggests that neoangiogenesis greatly contributes to the instability of atherosclerotic plaque. The microvessels in atherosclerotic plaque prominently express the integrin alpha(v)-beta(3) [1]. MRI is a promising technology that can provide noninvasive imaging with sub-millimeter resolution and high tissue contrast. In the present work, the noninvasive molecular imaging of plaque associated angiogenesis was assessed with a new low molecular weight non-peptidic RGD mimetic [2]. This alpha(v)-beta(3)-targeted molecule was grafted either to Gd-DTPA or to USPIO and the *in vivo* evaluation was performed on transgenic $ApoE^{-/-}$ mice.

Material and Methods. The RGD mimetic was obtained as described by Sulyok et al [2]. The molecule was grafted onto DTPA by reaction with pSCN-Bz-DTPA (Macrocyclics, Dallas, Tx, USA); DTPA-mimRGD was then complexed with $GdCl_3x6H_2O$. For grafting onto magnetic nanoparticles the dextran coating of USPIO was previously treated with epichlorhydrin. To assess the molecular imaging of atherosclerotic plaques, female C57Bl $apoE^{tm1Unc}$ mice were injected i.v. with one of the following contrast agents: 0.1 mmol/kg b.w. of Gd-DTPA-mimRGD; 60 µmol/kg b.w of USPIO-g-mimRGD or of USPIO. The animals were analyzed at 2 T (Oxford imaging system) and at 4.7 T (Bruker AVANCE-200 system). For the images acquired at 2 T, a half-birdcage RF resonator was adapted to the size of the mouse (25-mm wide and 40-mm length) [3]. The microimaging device was used for the images acquired at 4.7 T. The following MRI protocols were used on the two imaging systems: (A) RARE, TR/TE = 1050/9.6 ms, RARE factor = 4, spatial resolution = 0.09 mm (4.7T, Bruker AVANCE-200); (B) RARE, TR/TE = 3000/20 ms, RARE factor = 4, matrix = 256, spatial resolution = 0.09 mm) (4.7T, Bruker AVANCE-200); (C) fast SE, TR/TE = 2750/25-25 FOV = 23 mm, matrix = 256, spatial resolution = 0.09 mm (2T, Oxford imaging system) ; (D) TOF 3D, TR/TE = 13/3 ms, flip angle = 40°, matrix = 256x128x64, spatial resolution = 0.156x0.156x0.625 mm (2T, Oxford imaging system). Signal intensity (SI) values were measured within several regions of interest in the arterial wall of the aorta. The specific targeting of integrins was tested on Jurkat cells stimulated with phorbol myristate acetate (PMA). Plasma pharmacokinetics of USPIO-g-mimRGD were assessed on Wistar rats, healthy or with an inflammatory pathology known to involve integrin activation (hepatitis induced by concanavalin A).

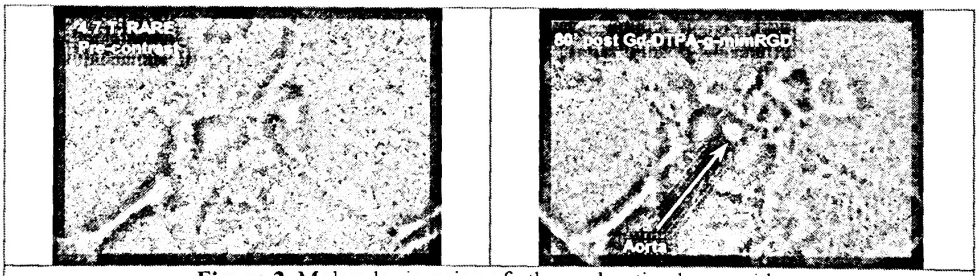

Figure 2. Molecular imaging of atherosclerotic plaque with Gd-DTPA-mimRGD (4.7 T, TR/TE = 1050/9.6 ms, spatial resolution = 0.09 mm).

Results. The images obtained at 4.7 T with Gd-DTPA-mimRGD have shown a striking enhancement (88%) of the signal at the level of atherosclerotic plaque 80 min post-contrast (Figure 1). The SI decrease induced by USPIO-g-mimRGD indicates that the contrast agent accumulates at the level of atherosclerotic plaque early after its administration (30 min) mainly around the blood vessel lumen. The specific interaction of USPIO-g-mimRGD with integrins expressed by Jurkat cells was inhibited by the peptide GRGD by 70%. A prolonged half-life of elimination was observed for USPIO-g-mimRGD (266 min) in pathological conditions associated to integrin expression as compared to healthy state (159 min).

Conclusion. The mimRGD-based contrast agents contribute to the high-resolution *in vivo* molecular imaging and quantification of unstable atherosclerotic lesions. The new contrast agents can find various applications for the MRI detection of angiogenesis-associated pathologies.

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Synthesis and Characterization of New Low Molecular Weight Lysine-Conjugated Gd-DTPA Contrast Agents suited for MR Angiography

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Introduction

The high molecular weight Gd-DTPA-conjugated polylysine compounds were extensively investigated in an attempt to develop optimal polymeric systems for blood pool imaging. However, the longer circulation time of these compounds is currently compromised by the presence of highly charged residues located on their backbone which are able to bind kidney cells as a result of co-operative interactions (1). In order to overcome this major drawback of the polylysine compounds, a new group of low molecular weight contrast agents were synthesized by conjugating the Gd-DTPA moiety directly to the $-NH_2$ group of Lys. The compounds were characterized by relaxometry and by their pharmacokinetic parameters evaluated in Wistar rats.

Material and methods

NMRD profiles were recorded on a fast field cycling relaxometer (Stelar, Italy). Transmetallation by zinc ions was evaluated by the decrease of the water longitudinal relaxation rate of buffered phosphate solutions containing gadolinium complex and $ZnCl_2$.(2) Blood pharmacokinetics were assessed on male Wistar rats (250 ± 20 g). Gd complexes were injected as a bolus through the femoral vein at a dose of 0.05 mmol/Kg b.w for $(Gd\text{-}DTPA)_6Lys_5$ and of 0.075 mmol/Kg b.w. for $(Gd\text{-}DTPA)_4Lys_3$. Blood samples were collected at different time delays. The gadolinium content of the blood samples was determined by relaxometry. A two-compartment distribution model was used to calculate the pharmacokinetic parameters (elimination half-life, $T_{e1/2}$, total clearance, Cl_{tot}, volume of distribution steady state, VD_{ss}).

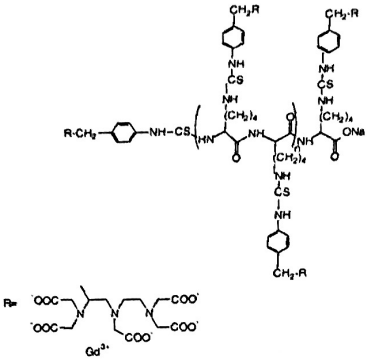
Results and discussion

Synthesis :

The peptides (Lys-Lys-Lys or Lys-Lys-Lys-Lys-Lys) reacted with an excess of p-SCN-Bz-DTPA ligand (Macrocyclics, Dallas, TX, USA) at pH 10 for 24h. The ligand was dialysed (cut-off membrane 1000) and then complexed with $GdCl_3.6H_2O$. The mass spectrometry confirms the structures.

Relaxometric characterization:

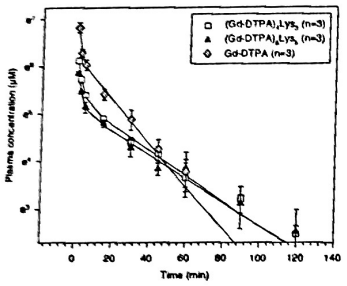
The relaxivity (310 K, 20 MHz) is equal to 5.7 and 6.4 $s^{-1}mM^{-1}$ for $(Gd\text{-}DTPA)_4Lys_3$ and $(Gd\text{-}DTPA)_6Lys_5$ respectively. The temperature dependence of the relaxivity at 20 MHz demonstrates that the water exchange is not a limiting factor. The higher relaxivities at 310 K as compared to Gd-DTPA are related to a longer rotational correlation time subsequent to a higher molecular weight of the molecule. These complexes do not interact with HSA. Transmetallation of the 2 complexes by Zn^{2+} ions shows a higher stability than the commercially used Gd-DTPA derivative.



Structure of complexes $(Gd\text{-}DTPA)_4Lys_3$ (n=1) and $(Gd\text{-}DTPA)_6Lys_5$ (n=2)

Pharmacokinetics

The pharmacokinetic parameters show a slightly prolonged blood residence time for $(Gd\text{-}DTPA)_4Lys_3$ ($T_{e1/2}$ = 26.4 min; Cl_{tot} = 7.3 ml/kg/min) and for $(Gd\text{-}DTPA)_6Lys_5$ ($T_{e1/2}$ = 28.5 min, Cl_{tot} = 6.9 mL/kg/min) as compared to Gd-DTPA ($T_{e1/2}$ = 14.9 min, Cl_{tot} = 8.66 mL/kg/min). The VD_β value (0.3 L/kg for both compounds) is moderately larger than that of Gd-DTPA (0.2 L/kg) and indicates some extravasation towards the interstitial space.



Conclusions

Even though the volume of distribution of the two compounds indicates a slow leakage into the interstitial space, their half-life in blood is slightly prolonged, which makes these compounds suitable as blood pool markers for MRI. The absence of positive molecular charge could limit the retention of the two compounds in kidneys, which is one of the major drawbacks of the high molecular weight polylysine contrast agents.

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Stealthy Magnetophages, a New Tool for Molecular Imaging

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Introduction

Phage display is a powerful tool in the context of molecular imaging. This technique allows to select, from a heterogeneous mixture of bacteriophages, displaying each a different peptide on their proteic wall, a vector that is subsequently coupled to a "contrastophore", i.e. USPIO for MRI. In our previous work [1], we have developed the concept of magnetophages, obtained by direct coupling of USPIO to phages isolated after the phage display procedures. We have shown that magnetophages can be used as in vitro molecular imaging contrast agents (MICA). However, in vivo they are taken up by the phagocytic cells of the reticulo-endothelial system (RES), particularly in the liver. This capture makes magnetophages unavailable to interact with the target. Therefore, "stealthy magnetophages" escaping the RES and showing a prolonged circulation time are suitable.

Materials and Methods

Stealthy magnetophages were obtained by covalent coupling of pegylated USPIO to the amino groups of the proteins of the phage wall. Stealthy magnetophages and non-stealthy magnetophages (i.e. nonpegylated USPIO), specific or not to the apoptotic marker phosphatidylserine (PS), were injected either to anesthetized control male mice or to mice bearing apoptotic liver. MRI images were acquired at 4.7 Tesla (Bruker Avance 200 system, Karlsruhe, Germany) using a T_2-weighted spin-echo sequence (TR/TE = 2000/20 msec, NE = 4, matrix 128x128, slice thickness = 2.5 mm, FOV = 6 cm). Intensities of regions of interest (ROI) defined in the liver were measured. Analysis of the images was based on the relative enhancement of the signal (RE) with respect to the pre-contrast image.

Results

Figure 1 shows that non-stealthy magnetophages, specific or not to PS, induce the RE decrease, probably due to their nonspecific internalization by the Kupffer cells of the apoptotic and healthy livers. On the contrary, figure 2 shows the decrease of RE only in the mice with apoptotic livers. This is conceivably correlated to the specific accumulation of stealthy magnetophages in apoptotic livers and not in healthy ones.

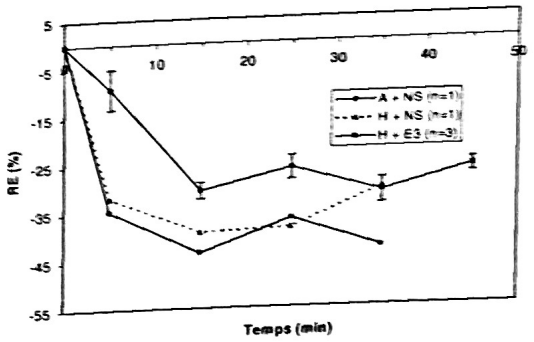

Figure 1 : Non specific magnetophages (NS) and magnetophages specific of PS (E3) injected in healthy (H) and apoptotic livers (A). Decrease of RE shows that magnetophages are accumulated in the liver.

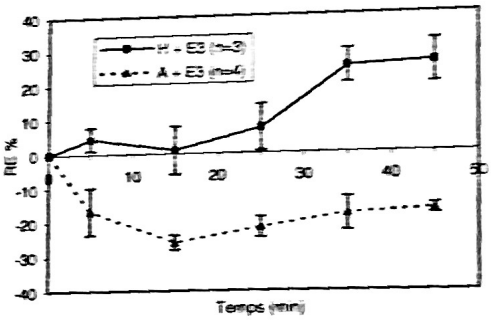

Figure 2 : Injection of stealth magnetophages specific of PS (E3) in apoptotic (A) and healthy livers (H). Decrease of RE in the first case suggests that magnetophages are accumulated in the apoptotic liver.

Conclusion

Stealthy magnetophages specific to PS can discriminate apoptotic liver from healthy one. They are invisible to the RES and can therefore be vectorized to their target. Stealth magnetophages can thus be used as in vivo molecular contrast agent.

References

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