Development of avidin-based positive contrast agents to detect tumor apoptosis using MRI

A. A. NEVES[1], A. KRISHNAN[1], M. KETTUNEN[1], D. HU[1], M. DE-BACKER[1], K. BRINDLE[1]
[1]DEPARTMENT OF BIOCHEMISTRY, UNIVERSITY OF CAMBRIDGE, CAMBRIDGE, CAMBS, United Kingdom

Introduction

Apoptosis, or programmed cell death is recognised as a prognostic indicator for the outcome of tumor therapy and therefore we have been developing a targeted MRI contrast agent that can be used to detect tumor cell apoptosis *in vivo*. The agent is based on the C2A domain of synaptotagmin I, which is a relatively small (14 kDa) protein that binds, in a Ca^{2+}-dependent manner, with nanomolar affinity to the phosphatidylserine (PtdS) that is externalised on the surface of cells undergoing apoptosis. Avidin is a biotin-binding protein with one of the strongest binding affinities in nature (K_d=10^{-15} M). We show here how biotinlyated C2A can be used in conjunction with avidin, which has been conjugated to gadolinium (Gd^{3+})-chelates, to produce a contrast agent that has the potential for signal amplification and improved avidity for PtdS on the surface of apoptotic cells. These are valuable properties, considering the intrinsically low sensitivity of MRI as an imaging modality.

Materials

A biotinylated version of C2A (biotC2A) was prepared using [sulfo-NHS-LC biotin] (Pierce), a biotinylation reagent with a spacer arm of 22.4 angstroms. Gd^{3+} was attached to chicken avidin (Sigma) using a pentavalent chelator [p-SCN-Bz-DTPA, Macrocyclics], which was reacted at a molar excess of 100-fold with the protein (Avidin-Gd). biotC2A and Avidin-Gd were combined at different molar ratios (n) in the range of 1 to 4 biotC2A to 1(Avidin-Gd), producing a series of macromolecular complexes [(biotC2A)n.(Avidin-Gd)], with molecular weights of 100-150 kDa. The agents were tested *in vitro* on apoptotic murine lymphoma cells (EL4). Cells (10^7) were incubated for 16 h with a chemotherapeutic drug (etoposide, 15 μM), washed and then incubated for 15 minutes, in the presence of 2 mM Ca^{2+}, with different ratios of the pre-mixed proteins [(biotC2A)n.(Avidin-Gd), n=0.8-3]. T_1 maps were calculated from inversion recovery gradient echo images (IR-FLASH, 15 TIs between 50ms and 15s, 15s delay between images, TR=5.5ms, TE=2ms), acquired at 9.4 T (Figure1). The complex [(biotC2A).(Avidin-Gd), n=1], was tested *in vivo* using the same tumor model. EL4 cells (10^6) were implanted subcutaneously in the flank of C57/BL6 mice and grown for 12-14 days, at which time they reached a volume of ~ 1 cm^3. The animals were then treated with etoposide (67 mg/kg) and cyclophosphamide (100 mg/kg) (i.p.) and 16 hours later the contrast agent was injected (i.v.) (0.5 mM, 200μl) and T_1 maps were calculated from inversion recovery gradient echo images (11 TIs between 50ms and 10s, 10s delay between images, TR=5.5ms, TE=2.5ms), acquired over the subsequent 24 hours (Figure 2).

Results

The T_1-relaxivities of the different [(biotC2A)n.(Avidin-Gd), n=1-3] complexes were in the range 60-75 mM^{-1} s^{-1} (at 9.4 T). The average number of Gd^{3+} ions incorporated on avidin was estimated at 8-10. A decrease in T_1 was observed *in vitro* with increasing molar ratios (n) of (biotC2A) to (Avidin-Gd) (Figure1). A decrease in tumor T_1 (Figure 2) was also observed *in vivo* 24 hours after injection of the contrast agent into an animal that had been treated with etoposide and cyclophosphamide.

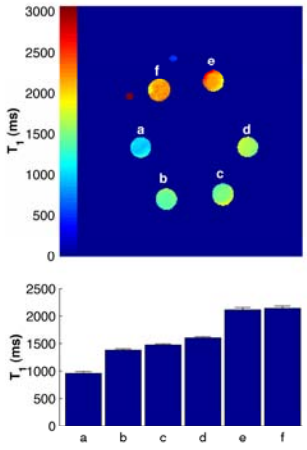

Figure 1 – T_1 relaxation times of [(biotC2A)n.(Avidin-Gd)] complexes binding to apoptotic EL4 cells. A larger T_1 change was observed with increasing biotC2A concentration. 10^7 apoptotic cells were incubated in the presence of a) biotC2A 72.8 μM, (Avidin-Gd) 24.0 μM, n=3; b) biotC2A 36.4 μM, (Avidin-Gd) 24.0 μM, n=1.5; c) biotC2A 18.0 μM, (Avidin-Gd) 24.0 μM, n=0.8; d) biotC2A 9.2 μM, (Avidin-Gd) 12.0 μM, n=0.8; e) biotC2A 0 μM, (Avidin-Gd) 60 μM; f) biotC2A 0 μM, (Avidin-Gd) 0 μM. The two capillaries contained buffer and (Avidin-Gd) 60 μM, respectively.

Discussion

Previous work from our lab[1] has demonstrated that C2A conjugated to superparamagnetic iron-oxide nanoparticles (SPIO), can be used to detect apoptotic cells, both *in vitro* and *in vivo*, in a murine lymphoma tumor model, using T_2-weighted MRI. Recently we have been concentrating on the development of smaller agents, based on Gd^{3+}-chelates, which give positive contrast in T_1-weighted images, the aim being to improve the sensitivity of apoptosis detection. We show here that relatively small complexes (150 kDa) based on biotC2A and Avidin-Gd can produce a relatively large increase in T_1 relaxation *in vitro* when bound to apoptotic cells (up to 2.5-fold) (Figure1). Preliminary studies using a 1-to-1 [(biotC2A).(Avidin-Gd)] complex (Figure 2), have shown that the contrast agent is retained in an apoptotic tumor 24 hours after injection, having mostly cleared from the surrounding tissue. Further complexes (n=2-4) will be tested *in vivo* to select an avidin-based positive contrast agent with adequate sensitivity, specificity and pharmacokinetic properties.

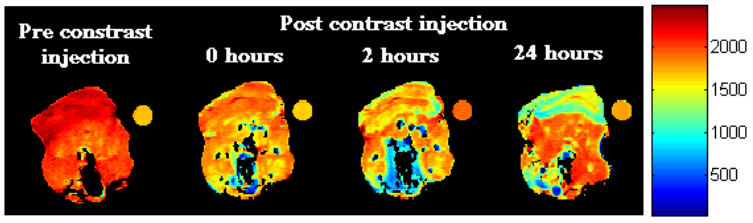

Figure 2 – T_1 maps of an apoptotic murine lymphoma before and after the injection of the contrast agent [(biotC2A).(Avidin-Gd)]. An average 1.6-fold increase in T_1 relaxation was observed in the tumor, 24 hours after injection. The average T_1 of the surrounding tissue, 24 hours post-injection, was similar to the T_1 of the same area pre-injection. T_1 expressed in milliseconds.

References

1. Zhao *et al.* (2001). *Nature Med* **7**, 1241-44.
2. Jung *et al.* (2004). *Bioconjugate Chem* **15**, 983-7.