Internalization of annexin A5-USPIOs by apoptotic cells

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Introduction

Apoptosis plays an important role in the etiology of a variety of cardiovascular diseases, including myocardial infarction, heart failure and atherosclerosis. Early detection of apoptosis in-vivo would allow for evaluation of disease treatment at an early stage. Annexin A5 is a protein that binds specifically to phosphatidylserine (PS) in the presence of Ca^{2+}. The phospholipid PS is exposed on the outer layer of the apoptotic cell membrane[1], and hence becomes accessible for binding to extra-cellular annexin A5. Therefore annexin A5 conjugated iron oxide particles enable the detection of apoptosis with MRI. The T_1, T_2 and T_2^*-reducing ability of iron oxide contrast agent depends on multiple parameters, including the cellular density of iron containing cells and the degree of iron oxide particle compartmentalization within the cell. For a given iron concentration homogeneously distributed particles were shown to induce shorter T_1 and T_2 relaxation times compared to iron oxide particles that are clustered for example in endosomes[2]. In contrast, particle accumulation in cellular compartments was shown to induce shorter T_2^* relaxation times compared to more homogeneously dispersed iron oxide particles[3]. The protein annexin A5 was recently shown to be internalized into endocytic vesicles by cells that are co-exposed to apoptotic stimuli, including anti-Fas[4]. Consequently annexin A5-USPIOs may be internalized in-vivo as well. Therefore the detection limit of apoptotic cells in-vivo with A5-USPIOs can be optimized by choosing the correct imaging sequence. In this study we investigated possible annexin A5-USPIO internalization both with MRI and transmission electron microscopy (TEM). For this purpose experiments were performed in which cells that were exposed to the contrast agents under conditions for which wild type annexin A5 would only bind to the outer leaflet of the cell membrane were compared to experiments in which phosphatidylserine-annexin A5-mediated pinocytosis would be observed.

Materials and methods

The T-lymphoma cell line Jurkat (ATCC) were grown in RPMI1640 medium at 37 °C in a humified atmosphere and 5% CO_2. Both annexin A5-functionalized and non-functionalized MACS microbeads were used as T_2 reducing contrast agents (Miltenyi Biotec, Auburn, CA). For the induction of apoptosis cells were harvested and resuspended to 10^6 cells ml^{-1} medium. RPMI1640 medium was used for binding experiments whereas Ca^{2+}-containing medium M199 was used for internalization experiments.

- *Binding:* Cells were treated for 3 hours with 200 ng ml^{-1} anti-Fas in order to induce apoptosis, harvested and respuspended in 2.5 mM Ca^{2+}-containing binding buffer to a final concentration of $2.5 \cdot 10^6$ cells ml^{-1}. Subsequently cells were left untreated or incubated with USPIOs or annexin A5-USPIOs (0.5 µg ml^{-1} Fe) for 15 minutes at room temperature.
- *Internalization:* USPIOs or annexin A5-USPIOs (0.5 µg ml^{-1} Fe) were added to medium M199. Additionally 200 ng ml^{-1} anti-Fas was added, and cells were incubated for 3 hours at 37 °C in a humified atmosphere and 5% CO_2.

Following the incubation periods of both experiments the cells were washed extensively either in Ca^{2+}-containing binding buffer or in a buffer that contained the Ca^{2+} chelator EDTA (5 mM), which is known to remove bound annexin A5-USPIOs from the cell membrane. Finally cells were fixed in a 2.5% gluteraldehyde solution, containing 2.5 mM Ca^{2+} or 5 mM EDTA respectively, and allowed to settle to a loosely packed cell pellet in a 250 µl tube overnight at room temperature. T_2 relaxation times of the cell pellets were measured at 6.3 T. The spatial location of the annexin A5-USPIOs that provided the apoptotic cell pellets with reduced T_2 values was determined with transmission electron microscopy (TEM). For TEM cells were postfixed in 1% osmium tetroxide solution, dehydrated and embedded in epoxy resin. Additional EDX spectrometry was performed to confirm the presence of iron oxide in areas of the TEM images where annexin A5-USPIOs were visually identified.

Results

Annexin A5-USPIOs were shown to increase the relaxation rate R_2 (T_2^{-1}) of apoptotic cell pellets in the presence of Ca^{2+} for both the binding and the internalization experiments, whereas R_2 values remained unaffected by incubation with non-functionalized USPIOs (Fig1). Removal of Ca^{2+} by extensive washing in an EDTA-containing buffer reduced the R_2 values of cells that were incubated with the annexin A5-USPIOs after stimulation with anti-Fas to control levels (Fig1. *Binding*). R_2 values of cells that were co-incubated with annexin A5-USPIOs and anti-Fas were also decreased by removal of Ca^{2+}, however R_2 values did not return to control levels (Fig1 *Internalization*). TEM images of cells that were incubated with annexin A5-USPIOs following stimulation with anti-Fas and subsequently washed in binding buffer showed binding of the annexin A5-USPIOs to the cell membrane (Fig2 *left*). On the other hand internalized annexin A5-USPIOs were found for cells that were incubated with annexin A5-USPIOs in combination with anti-Fas (Fig2 *right*). EDX spectra displayed large iron peaks in the areas where annexin A5-USPIOs were identified on the TEM images, whereas no iron peaks were found in the surrounding areas (Fig3).

Discussion and conclusion

Annexin A5-USPIOs were shown to be internalized into vesicles if cells are co-exposed to annexin A5-USPIOs and apoptotic stimuli, indicating that phosphatidylserine-annexin A5-mediated pinocytosis is not restricted by conjugation to high molecular weight contrast agents. In vitro results presented here suggest that these contrast agents can be expected to be internalized in-vivo as well. Consequently, when large number of iron oxide particles are internalized apoptosis may be detected at an earlier stage in T_2*-weighted images compared to T_2-weighted images. Further research is required to quantify the effect of the spatial location of the annexin A5-USPIOs on the ratio of relaxivity r_2^* to r_2.

References
1. Fadok V.A.*et al.*, *Nature*, 4:405(6782):85-90 (2000).
2. Billotey C. *et al., Magnetic Resonance in Medicine,* 49:646-654 (2003).
3. Bowen C.V. *et al., Magnetic Resonance in Medicine,* 48:52-61 (2002).
4. Kenis H. *et al., The Journal of Biological Chemistry*, 10;279(50):52623-9 (2004).

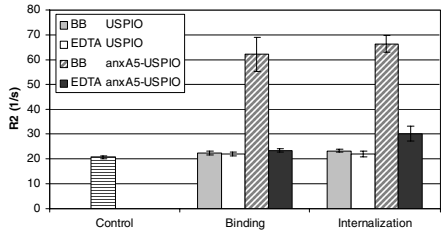

Figure 1: Relaxation rates R_2 (s^{-1}) of untreated cells (control) or cells that were incubated with USPIOs or annexin A5-USPIOs after stimulation with anti-Fas (Binding) or in combination with anti-Fas (Internalization). Following incubation with the contrast agents the cells were either washed in a Ca^{2+}-containing binding buffer (BB) or in a EDTA-containing buffer (EDTA). Error bars represent the error on the R_2 values from the T_2 fitting procedure.

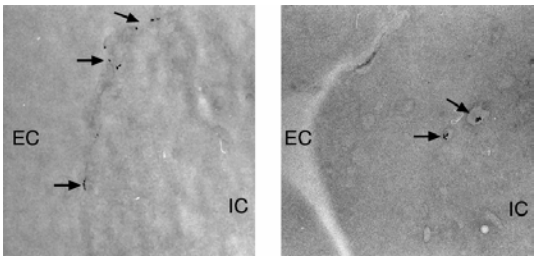

Figure 2: TEM images (6300x) of cells that were incubated with annexin A5-USPIOs after stimulation with anti-Fas (left) or in combination with anti-Fas (right). The cytosol (IC) can be distinguished from the extracellular compartment (EC). Annexin A5-USPIOs are indicated by the arrows.

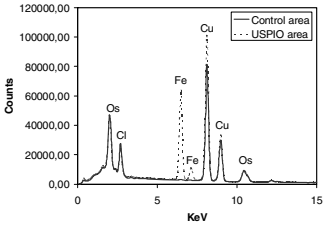

Figure 3: EDX spectrum of an area where annexin A5-USPIOs appeared to be accumulated (dotted). A control spectrum was obtained in the surrounding area (solid).