Targeting phosphatidylserine by phage display to image apoptosis

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INTRODUCTION

The programmed cell death, or apoptosis, is one of the most explored subject of the modern biology. Morphologically, it corresponds to a progressive withdrawal of the cell, with chromatin and cytoplasm condensation, followed by a particular DNA fragmentation. At the end of this process, cellular fragments, known as apoptotic bodies, are formed. They are phagocyted by the neighbouring cells with no inflammatory reaction.

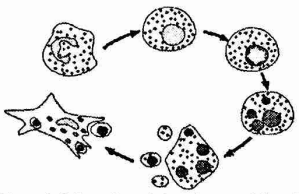

Figure 1: Schematic evolution of an apoptotic cell

Apoptosis is a spontaneous process of cell elimination. Its failing or over-activation are responsible for a broad range of pathologies (carcinogenesis, neurodegenerative diseases, auto-immune diseases, graft rejection,...)

Phosphatidylserine (PS) seems to be an interesting candidate as marker of apoptosis. Its targeting to its natural ligand annexin V has indeed already been mentioned both *in vitro* **[1]** and *in vivo* **[2].** In this work, we have used the technique of phage display **[3]** with the aim to isolate peptides of high affinity for PS. The final goal is the coupling of those peptides to a magnetically active entity to get a specific contrast agent for the MRI detection of apoptotic cells.

SUBJECTS AND METHODS

Liver perfusion

Mice livers were isolated and perfused (37°C) at a constant flow rate 1.5 ml/min with a Krebs-Henseleit solution saturated with carbogen (95%O_2/5%CO_2).

Induction and confirmation of apoptosis

Liver apoptosis has been induced in BALB/cByJIco mice by intravenous injection of 10 µg of purified hamster anti-FAS antibody (Jo2, Becton-Dickinson, Benelux) **[4]**. The presence of apoptotic hepatocytes has been confirmed by TUNEL assay **[5]**.

Biopanning of phage display libraries in perfused mice livers

The phage display library (linear 6-mer) was first incubated with a healthy liver during 30 minutes. The phages remaining in the liver perfusate were amplified by infection of *E. coli* TG1. This step has been repeated 3 times. The same protocol was used for the selection on apoptotic liver. The phages of interest, which are remaining in the liver, are eluted by decreasing the pH.

In vitro target binding assays

The libraries obtained after all the selection rounds and each individual phage clone were tested for their affinity for PS and phosphatidylcholine (PC) by ELISA technique. The phages were incubated with phospholipids immobilized on ELISA plates. OD was measured at a wavelength of 450 nm.

Determination of apparent affinity constants

In order to determine the apparent affinity constant (Ka,app), binding experiments were performed by ELISA as described above.

Competition ELISA with annexin V

Competition ELISA was carried out as described with final phages concentration corresponding to the halfmaximal binding in the fixation curve. Serial dilutions of annexin V were incubated with PS during 30 min. Then, phages were incubated during 1h30 at 37°C in the presence of the competitor.

DNA sequencing

PCR amplification of the peptide-coding inserts was peformed on isolated phage ssDNA using the primer: $^{3'}$GGAGTATGTCTTTTAAGT$^{5'}$.

RESULTS

Confirmation of apoptosis

The TUNEL assay has confirmed that about 80% of hepatocytes were apoptotic two hours after anti-FAS injection.

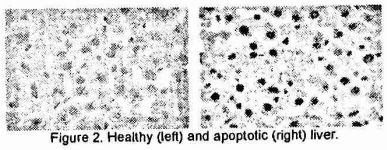

Figure 2. Healthy (left) and apoptotic (right) liver.

Binding assay

As expected during the selection on healthy liver, the affinity of phages for the target (PS) decreases since the phages interacting with healthy cells are progressively removed from the library. During the selection on apoptotic liver, the affinity for PS increases confirming that the library is enriched in phages specific for PS.

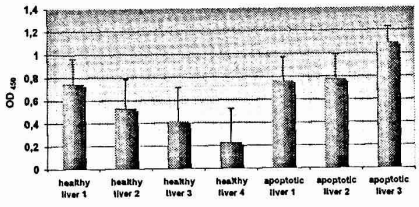

Figure 3. Evolution of the phage libraries affinity for phosphatidylserine

The individual affinity of selected phages was estimated for PS and PC by ELISA technique in order to eliminate phages having a higher affinity for PC since they could also interact with healthy cells. The phages presenting the largest ratio between affinity to PS and affinity to PC were selected for the sequencing.

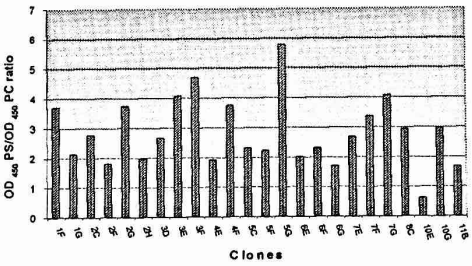

Figure 4. Ratio between PS and PC affinity of each phage clone.

Apparent affinity constant

Fig. 6 presents the results obtained on four of the selected phages. From the fixation curves of each selected clone, it seems that the clone with the highest affinity is the clone 3E.

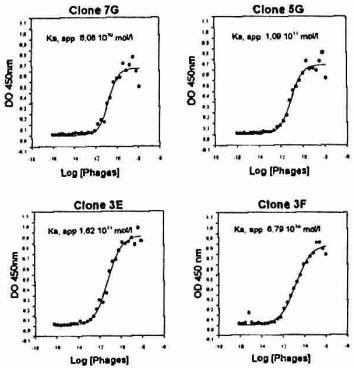

Figure 6. Fixation curves of the selected clones.

Competition ELISA with annexin V

Competition experiments show an OD decrease corresponding to a decrease of the phages fixation, when annexin V concentration increases.

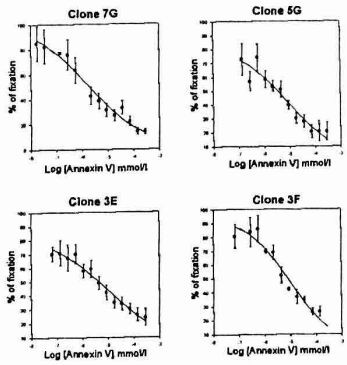

Figure 7. Competition curves of the selected clones.

Peptide sequences

No sequence homology could be observed between the different clones. This can be explained by the fact that the exposure of the phages to a whole organ leads indeed to the selection of a broad spectrum of phages interacting with apoptotic structures. Nevertheless, one of them presents a short homology sequence with a region of annexin V known to interact to PS.

SVSNQY : Peptide sequence

RSVSHLRKV :Annexin V fragment

However this peptide does not have the highest affinity PS as compared to clones 3E, 3F, 5G and 7G.

DISCUSSION

In our model, apoptotic hepatocytes are used as PS support. These cells present also other molecules specific of apoptosis. Therefore, libraries obtained after successive selection rounds contain phages interacting with PS and/or some other apoptotic structures. Affinity constants of the four selected clones are high and close to each other.Competition ELISAs have confirmed the existence of specific interaction.

CONCLUSION

The peptide of the clone with the highest affinity (3E) will be coupled to a magnetically active centre to generate a new contrast agent specific of apoptosis. Due to their high relaxivity, superparamagnetic particles of iron oxide (USPIO) are very suitable magnetic agents for this kind of application. The peptide coupled to USPIO could be used for *in vivo* or *in vitro* tests, aiming at the detection of apoptotic cells, or more simply, at the detection of PS.

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