Pro-metastatic effect of *Candida albicans* mannoproteins on hepatic endothelial cells

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Introduction

Candida albicans is a dimorphic fungus that is usually found in humans as commensal microorganism. However, it can become an opportunistic pathogen, specially in the case of immunocompromised patients, causing disseminated infections. When this yeast reaches the bloodstream, it is cleared mainly by the liver. Once in the liver blood vessels, *C. albicans* binds to hepatic sinusoidal endothelial (HSE) cells, inducing a pro-inflammatory process. In consequence, pro-inflammatory cytokines are secreted and adhesion molecules expressed. In healthy individuals, these cytokines recruit innate immune cells that help the clearance of the infection. In the case of cancer patients, on the contrary, immune cells are present in low levels, and these adhesion molecules may enhance the adherence of circulating tumor cells, leading to liver metastasis (Fig. 1).

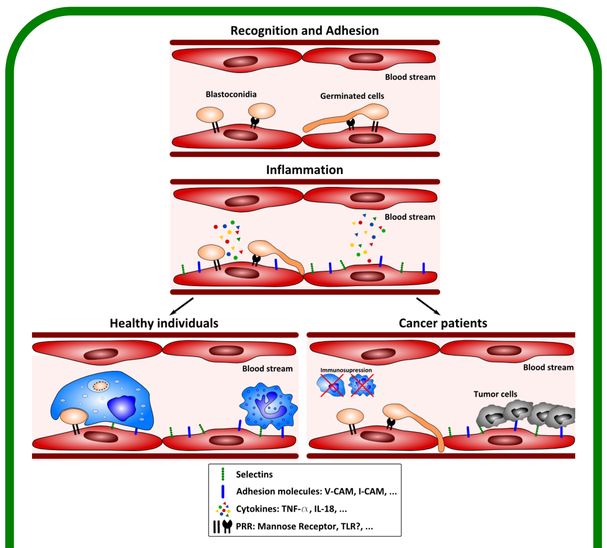
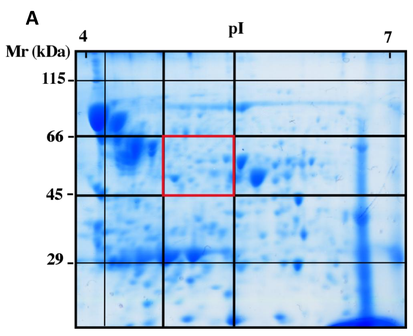


Fig 1. Increased tumor adhesion in cancer patients as consequence of a pro-inflammatory response of HSE cells after stimulation with *C. albicans*. (Ramirez-García *et al.*, 2016)

Previous results

To simulate a metastatic process, we developed an *in vitro* adhesion model of B16 melanoma (B16M) cells to HSE (Fig. 4), and we found that HSE stimulation by *C. albicans* led to an increase of B16M adhesion. In order to characterize the fungal molecules involved in this process, yeast proteome was divided in different mannoprotein fractions and we identified the proteins belonging to the fraction that induced the highest tumor cells adhesion. Among them, four proteins (in red) were chosen because of their immunogenic activity (Fig. 2 A-B). Additionally, in order to check the role of IL-1β cytokine and its receptor, which seem to be important in this process, Western Blot analysis using an anti-IL-1β antibody was performed on the same fraction. Interestingly, one protein, Kre9, was identified in this way (Fig. 2 C).



B

Name	Full name
PDI1	Protein disulfide isomerase
APE3	Aminopeptidase Y
ILV5	Mitochondrial ketol-acid redcuto-isomerase
TUF1	Elongation factor Tu
ACT1	Actin
ENO1	Enolase
ADH1	Alcohol dehydrogenase
DOR14	6-phosphogluconate dehydrogenase
FBA1	Fructose-biphosphate aldolase
PGK1	3-Phosphoglycerate kinase
ADH1 + MPG1	Alcohol dehydrogenase 1 + GDP-mannose pirophosphorilase
SFA1	Glutathione-dependent formaldehyde dehydrogenase
QCR2	Ubiquinol-cytochrome c reductase

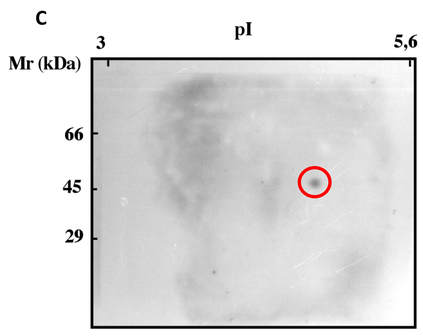


Fig. 2. A) In red, the mannoprotein subfraction that induces the highest increase in tumor cell adhesion to HSE cells. **B)** Proteins belonging to the mannoprotein subfraction between 45 to 66 KDa (Ramirez-Garcia *et al.*, 2011); in red the proteins chosen for this study. **C)** Western blot of the mannoprotein fraction using anti-IL-1β antibody, in which Kre9 was recognized.

Methods

1. PRODUCTION OF RECOMBINANT PROTEINS AND MONOCLONAL ANTIBODIES

The five proteins above-mentioned were selected for further study and were obtained as recombinant proteins in an *Escherichia coli* model (Fig. 3): Alcohol dehydrogenase (Adh1), Mitochondrial ketol-acid reducto-isomerase (Ilv5), Enolase (Eno1), Ubiquinol-cytochrome c reductase (Qcr2) and Kre9. Two of these proteins, Adh1 and Kre9, were selected for more detailed studies and monoclonal antibodies (MAb) against them were produced by two different enterprises; Abyntek (Parque Tecnológico de Zamudio, Bizkaia) and Abmart (Shanghai, China), respectively.

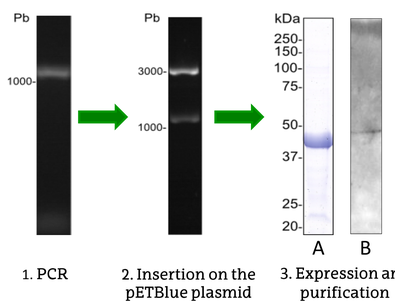


Fig. 3. Clonation in an *E. coli* model: **1**. Amplification of the target gene by PCR. **2**. Digestion of the plasmid pETBlue2 containing the gene insert. **3**. A) SDS-PAGE and Blue Coomassie staining of the expressed and purified protein using His Trap™ FF crude columns and B) Western blot of the purified protein using anti-HIS antibody.

2. ADHESION OF B16M TO HSE

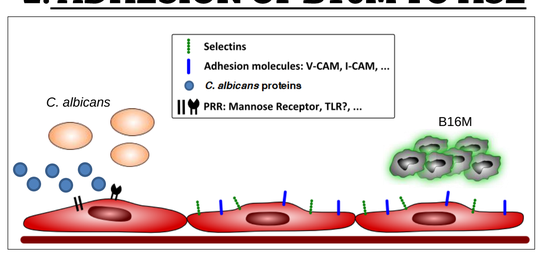


Fig. 4. Scheme of the B16 M tumor cell adhesion *in vitro* model.

HSE cells were incubated with *C. albicans* cells or with 2 ng/ml of recombinant proteins for 18 h. After that, fluorescently stained B16M cells were added, and the increase of adhesion, compared to untreated control, was measured (Ramirez-Garcia *et al.*, 2013).

Influence of *C. albicans* recombinant proteins in B16M adhesion to HSE

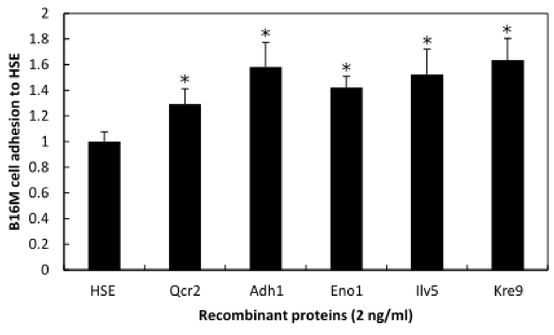


Fig. 5. Adhesion of B16M cells to HSE cells in presence of 2 ng/ml of the recombinant proteins for 18 h. *p ‹ 0.05

HSE cells cells were incubated with recombinant proteins for 18 h, and, as previously mentioned, after that incubation period B16M tumor cells were added to the cells. The adhesion was measured and compared to the non-stimulated control. All the proteins induced an increase in relative adhesion, being the highest increase the one induced by incubation with Adh1 and Kre9 proteins (Fig. 5).

Effect of anti-Kre9 and anti-Adh1 MAbs on B16M adhesion to HSE

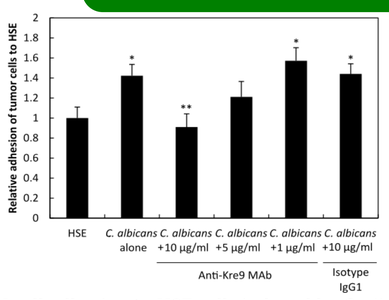


Fig 6. B16M cell adhesion to HSE cells induced by *C. albicans* pre-incubated with different concentrations of anti-Kre9 Mab and the isotype control. *Differences with HSE control, ** Differences with *C. albicans* alone. p ‹ 0.05

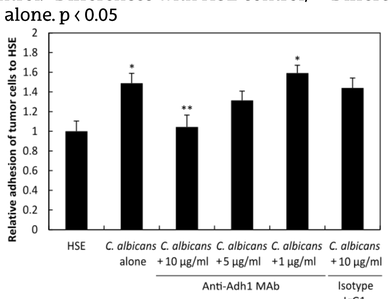


Fig. 7. B16M cell adhesion to HSE cells induced by *C. albicans* pre-incubated with different concentrations of anti-Adh1 Mab and the isotype control. *Differences with HSE control, ** Differences with *C. albicans* alone. p ‹ 0.05.

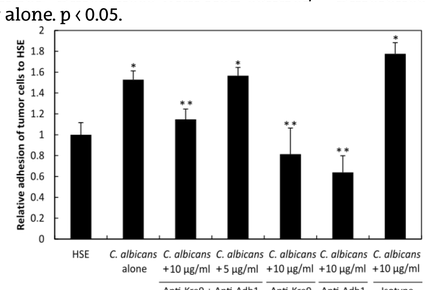


Fig. 8. B16M cell adhesion to HSE cells induced by *C. albicans* pre-incubated with both anti-Kre9 and anti-Adh1 MAbs and the isotype control. *Differences with HSE control, ** Differences with *C. albicans* alone. p ‹ 0.05.

C. albicans was pre-incubated for 30 minutes at 37ºC with different concentrations of anti-Kre9 or anti-Adh1 MAbs (Fig 6 and 7) and then the excess of Mab was discarded by centrifugation. After incubation of the pre-incubated fungus with the endothelial cells for 18 h, tumor cell adhesion was measured. An IgG1 isotype control was added in order to assure that the effect seen was due specifically to the antibodies tested.

In both cases, the highest antibody concentration used, 10 µg/ml, was the one inducing the highest inhibition of tumor cell adhesion, almost to the level of the non-stimulated control.

The effect of combination of both antibodies was also studied. As seen in figure 8, combination of 10 µg/ml of MAbs led to an inhibition of cell adhesion, but not as much as the one induced by each antibody on its own.

The isotype control did not reduce the tumor cell adhesion, showing that this effect is specific to the developed MAbs.

Anti-Kre9 MAb specifically recognizes C. *albicans* hyphae

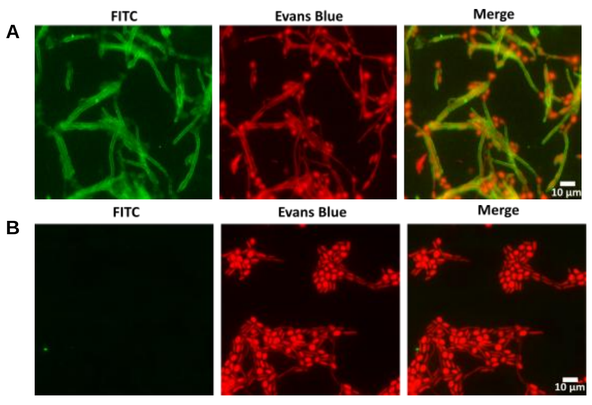


Fig. 9. Indirect immunofluorescence using anti-Kre9 MAb. FITC shows the recognition of the antibody. Whole cells are visualized with Evans Blue. Both images are overlapped in merge. **A)** *C. albicans* hyphae are recognized by the antibody. **B)** *C. dubliniensis* is not recognized by the antibody.

The ability to form true hypahe is considered to be a major virulence factor of *C. albicans*. In order to see if Kre9 mannoprotein is found in this fungus hyphae, *C. albicans* and *C. dubliniensis* (the only other *Candida spp.* able to form true hyphae) yeasts and hyphae were incubated with anti-Kre9 antibody.

As shown in figure 9, *C, albicans* hypahe were strongly recognized by the antibody, whereas yeasts were only slightly stained. Furthermore, the antibody did not bind neither to yeasts nor to *C. dubliniensis* hypahe.

Conclusions

- *C. albicans* recombinant proteins Adh1, Ilv5, Eno1, Qcr2 and Kre9 induce an increase in B16M adhesion to HSE cells.
- Pre-incubation of *C. albicans* with anti-Kre9 and/or anti-Adh1 antibodies reduces the adhesion of B16M cells to HSE.
- Anti-Kre9 antibody specifically recognized *C. albicans* hyphae and not *C. dubliniensis*.
- **In depth research of *C. albicans* mannoproteins may lead to the discovery of new diagnostic or terapeutic tools as shown by the results here presented.**

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