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Metallochelating liposomes with associated lipophilised norAbuMDP as biocompatible platform for construction of vaccines with recombinant His-tagged antigens: Preparation, structural study and immune response towards rHsp90

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ABSTRACT

Hsp90-CA is present in cell wall of *Candida* pseudohyphae or hyphae — typical pathogenic morphotype for both systemic and mucosal *Candida* infections. Heat shock protein from *Candida albicans* (hsp90-CA) is an important target for protective antibodies during disseminated candidiasis of experimental mice and human. His-tagged protein rHsp90 was prepared and used as the antigen for preparation of experimental recombinant liposomal vaccine. Nickel-chelating liposomes (the size around 100 nm, PDI ≤ 0.1) were prepared from the mixture of egg phosphatidyl choline and nickel-chelating lipid DOGS-NTA-Ni (molar ratio 95:5%) by hydration of lipid film and extrusion methods. New non-pyrogenic hydrophobised derivative of MDP (C18-O-6-norAbuMDP) was incorporated into liposomes as adjuvans. rHsp90 was attached onto the surface of metallochelating liposomes by metallochelating bond and the structure of these proteoliposomes was studied by dynamic light scattering, AF microscopy, TEM and GPC. The liposomes with surface-exposed C18-O-6-norAbuMDP were well recognised and phagocyted by human dendritic cells *in vitro*. *In vivo* the immune response towards this experimental vaccine applied in mice (i.d.) demonstrated both TH1 and TH2 response comparable to FCA, but without any side effects. Metallochelating liposomes with lipophilic derivatives of muramyl dipeptide represent a new biocompatible platform for construction of experimental recombinant vaccines and drug-targeting systems.

1. Introduction

Candidiasis, the infection caused by the *Candida* species, is considered an important medical problem in both developing and developed countries. The systemic or deep form of candidiasis occurs predominantly as a consequence of some high-risk medical procedures, immunosuppressive therapy, and aging. It affects organs such as the brain, liver, spleen, lungs, eyes, heart, and kidneys leading to abscesses formation and organ failure associated with mortality in approx. 50% of all cases, irrespective of the administration of intensive antifungal therapy [1]. Humoral immunity provides substantial protection against *Candida* growth [2,3]. Protective antibodies have direct candidastatic activity with no direct need for cellular help. They can act by opsonisation, neutralisation of extracellular virulence factors (proteases, immunomodulating fungal products such as polysaccharides), inhibition of the *Candida* adherence to host tissue, inhibition of yeast-to-hyphae transition, and by direct fungicidal activity [4–6]. The switch from the yeast to a hyphal morphotype is one of the most important factors of *Candida* virulence and survival. Up to now, several *Candida* specific antigens have been identified that are considered to be a target for protective antibodies. Beside surface

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exposed β-(1,2)-mannotriose or stress mannoproteins, inner cell wall β-(1,3)-glucans, and secreted aspartyl proteinases, heat shock protein 81 kDa (Hsp90) was identified as the protective antigen [3,4,6–8]. The *C. albicans* Hsp90 protein is localized only in the cell wall of budding yeasts and hyphae but not in the cell wall of non-proliferating yeasts [9]. It is essential for the *Candida* viability [10]. Hsp90 was structurally characterised in 1995 [10]. It is functionally involved in re-folding of mitogen-activated protein kinases, enzymes essential for the cell wall synthesis and *Candida* morpohogenesis. A knock-out of *Candida* MAP kinase mkc1 causes a) a lower growth rate, b) a loss of viability during cultivation at 42 °C, and c) a decreased thickness of the cell wall and higher vulnerability to lytic enzymes [9]. Hsp90 was suggested to be a protective antigen following the detection of high levels of Hsp90-specific antibodies in sera of patients that recovered from systemic candidiasis in contrast to those which succumb [11]. These antibodies recognised Hsp90-breakdown heat-stabile C′ terminal fragment of 47 kDa which is released from living *Candida* cells after hydrolytic digestion of Hsp90. Hsp90 was confirmed to be a protective antigen in an animal model of disseminated candidiasis [9,12]. Due to the strong evolutionary conservation of Hsp90 sequence there are speculations about the possibility that Hsp90 administration could elicit autoimmune reaction or tolerance. Nevertheless, until now there is no direct evidence of such effects [12–14].

The advent of recombinant vaccines based on both DNA and recombinant protein antigens has recently forced researchers to consider alternative vaccine designs and deal with the low immunogenicity of constructs with a corresponding need for strong and biologically acceptable adjuvants to accompany antigenic components. For the construction of prophylactic as well as therapeutic vaccines against *Candida* we have been considering the use of metallochelation liposomes for simple coupling of His-tagged recombinant Hsp90 (rHsp90). Liposomes are potentially very useful for the construction of vaccination systems given their ready biodegradability and versatility as carriers for varieties of molecules having different physico-chemical properties (such as size, hydrophilicity, hydrophobicity, or net electrical charge). Liposomes also offer the possibility for simultaneous association or entrapment of more than one type of molecule. Of particular interest to us has been the possibility the co-association of hydrophilic or lipophilic adjuvants (e.g. monophosphoryl lipid A [MPL A], CpG oligonucleotides, muramyl dipeptide (MDP) and/or MDP analogues) with soluble or membrane protein antigens, ligands for the targeting of specific receptors on antigen-presenting cells [15]. In this way, liposomes become transformed into multifunctional platforms for vaccination that represent real multifunctional vaccination particles.

This manuscript describes the preparation of rHsp90 proteoliposomes, the study of their structure by DLS, GPC, TEM and AFM as well as *in vivo* immune responses to rHsp90 proteoliposomes or prototype rHsp90 vaccination particles that also comprise a lipophilic derivative of muramyl dipeptide (MDP) known as C-18-O6-norAbuMDP (MT03). This is the first time that such a defined synthetic adjuvant has been used to construct a recombinant protein vaccination nanoparticle.

2. Materials and methods

DiO C18 (3,3′-dioctadecyloxacarbocyanine perchlorate) was purchased from Molecular Probes (Invitrogen, Carlsbad, CA). Lipids: egg phosphatidylcholine (EPC, purity of 99%), 1,2-Dioleoyl-sn-Glycero-3-[N(5-Amino-1-Carboxypentyl)iminodiacetic Acid] Succinyl (Nickel Salt) (DOGS-NTA-Ni), lyssamine-rhodamine phosphatidylethanolamine and fluorescein phosphatidylethanolamine were purchased from Avanti Polar Lipids (Birmingham, AL). 20 nm membrane filter Anotop 10 and 0.2 μm Anotop 10 LC were purchased from Whatman (Maidstone, UK). *All chemicals*, unless otherwise specified, were from Sigma (St. Louis, MO).

2.1. Synthetic MDP derivative C-18-O6-nor-AbuMDP (MT03)

For the incorporation into the lipid structures such as liposomes, the normuramyl glycopeptide was modified by stearoyl substituent on O-6 position of the sugar part of the molecule (Fig. 1). The syntheses and the confirmation of the structures are described by Ledvina and co-workers (Ledvina M., Turánek J., Miller A.D., Hipler K., Compound (Adjuvants): PCT appl., WO 2009/11582 A2, 2009, US 12/922,663).

2.2. Preparation, purification, and characterisation of rHsp90

Full length *Hsp90* cDNA was isolated from a clinical isolate of *C. albicans* verified at the Department of Microbiology, Faculty of Medicine and Dentistry, Palacky University in Olomouc, Czech Republic as reported before [12]. The cDNA was analyzed by sequencing and BLAST analysis, which confirmed the identity with *C. albicans Hsp90* (*GenBank* Accession No. X81025). The recombinant Hsp90 protein (rHsp90) was expressed as a fusion protein with both N` and C`-terminal His tag in *E. coli* [12] and further purified using Ni-NTA affinity agarose column under native conditions (Qiagen, Hilden, Germany) as reported elsewhere [16]. Endotoxin was removed by successive two-phase separation with Triton X-114 as described elsewhere [16]. The entire procedure was repeated until the endotoxin level was below 0.25 EU per 1 mg of protein.

2.3. SDS-PAGE, western blot, and MALDI-TOF analyses

Protein samples were separated on 10% SDS-PAGE and stained with Coomassie Blue R-250 or blotted to PVDF membrane (BioRad, Hercules, CA) and developed with HRP-conjugated Anti-T7 (Merck, Darmstadt, Germany) diluted 1:5,000 in SuperBlock plus 0.05% Tween 20 (SB-T) followed by detection with the SuperSignal West Pico reagents (Pierce) and by exposition on an X-ray film (Kodak, Rochester, NY). Protein identity was confirmed by peptide mass fingerprinting of SDS-PAGE-resolved rHsp90 preparation using a Microflex LRF20 MALDI-TOF mass spectrometer (Bruker Daltonik, Bremen, Germany) as described before in detail [17,18]. Protein identification from the obtained mass spectra was achieved using the Mascot search engine (Matrix Science, London,

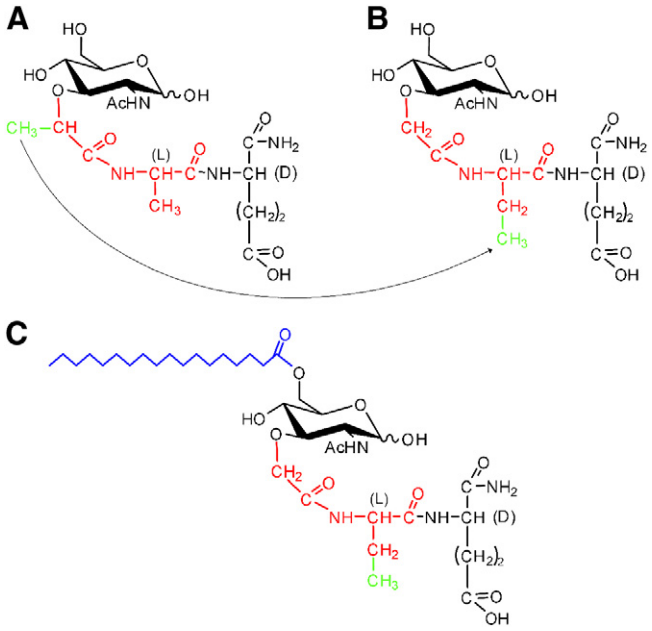

Fig. 1. Structural formula of *C-18-O6-nor-AbuMDP* (MT03) and MDP. Structural changes in the molecule of MT03 are marked in red and green. Hydrophobic accessory domain (stearoyl) is marked in blue. A) MDP; B) NorAbuMDP; C) C-18-O6-nor-AbuMDP.