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Short Communication

Melittin-loaded immunoliposomes against viral surface proteins, a new approach to antiviral therapy

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ABSTRACT

In this study, melittin, a well-characterized pore-forming lytic amphiphilic peptide susceptible to be vehiculized in lipid membranes, has been utilized to study their antiviral properties. For this purpose, an assay based on melittin loaded-immunoliposomes previously described by our group was adapted to antiviral purposes by means of monoclonal antibodies targeting the surface G glycoprotein of the fish viral haemorrhagic septicemia rhabdovirus (VHSV). We also studied the antiviral action of these immunoliposomes *in vitro* and the results showed that they are capable of inhibiting the VHSV infectivity by 95.2% via direct inactivation of the virus. Furthermore, the inhibition of the infectivity when treatments were added at different times post-infection and the analysis of the infection foci sizes suggested altogether that they also act by reducing the VHSV spread in cell culture and by killing the infected cells which express the G glycoprotein in their plasmatic membranes.

1. Introduction

Despite the well-known problems with virus diseases worldwide, efficient therapeutic agents against viral infections are currently insufficient and limited. In many cases they fail because of severe side-effects, drug-resistant mutant appearances, etc.

Due to the above-mentioned facts, new approaches are being developed in order to find safer and more effective antiviral agents. In this regard, research on antimicrobial peptides (AMPs), a component of the non-specific immune system, is becoming an interesting alternative since the number of known AMPs with antiviral activity is constantly increasing (Lai and Gallo, 2009). AMPs are cationic peptides isolated from a wide range of organisms that exert microbicidal activity against a broad spectrum of microorganisms, including both enveloped and non-enveloped viruses (Ganz, 2001; Zasloff, 2002; Patrzykat and Douglas, 2005). In addition, they play other potential roles in innate immunity, acting not only directly on the pathogen microorganisms but also on the host cells, which makes difficult the development of resistance against them. Although they have much more affinity to the microbial membranes, to use them as therapeutants, the delivery mechanisms must be improved so as to avoid possible side-effects and to increase their effectiveness.

AMPs can be classified in three structural groups: (i) linear/α-helical, (ii) disulfide stabilized/β-sheet or (iii) extended helices and cyclized loops. In any of these groups, most of AMPs share a common amphiphilic structure that contributes to a general mechanism of action based on their interaction with the lipid cell membranes of pathogens such as bacteria and/or enveloped viruses (Jelinek and Kolusheva, 2005; Shai, 2002). This interaction causes direct destabilization/permeabilisation of the target pathogen lipid membrane, an essential dynamic structure fairly conserved in microorganisms, what makes resistance to AMPs difficult to be developed by the pathogens (Zasloff, 2002; Hancock and Scott, 2000). However, although AMPs present more affinity for the commonly negatively charged membrane of pathogens, the complete elimination of the pathogens requires high doses that may contribute to unspecific binding or dose exceed. Thus, to develop local and enhanced effects, AMPs should be successfully delivered into their target sites, for instance by using promising nano-strategies such as liposomes.

In the liposome field, newer improved therapies based on the use of immunoliposomes containing chemotherapeutic agents are emerging. For instance, the conjugation of complete or fragmented antibodies to liposomes has resulted in the next generation of delivery drugs (Noble et al., 2004; Park et al., 1997). Thus several anticancer therapies targeting members of the epidermal growth factor receptor, such as the protooncogene HER2 (ErbB2),

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have been already developed. We have reported the specific targeting of breast cancer cells using anti-HER2 immunoliposomes (Barrajon-Catalan et al., 2010). Moreover, the strategy has been successfully extended to other overexpressed membrane receptors such as the epithelial cell adhesion molecule (EpCAM) (Barrajón-Catalán et al., 2011).

Immunoliposomes may also support an alternative for antiviral therapy which brings additional advantages compared to more traditional cancer therapies. First, viruses show a varied repertoire of target proteins for antibody designs. Second, antibodies against viral proteins are more specific compared to cancer cells receptors because cancer and normal cells share most receptor proteins and only differ in their expression level. Moreover, immunoliposomes may be able to target not only the virus but also infected cells bearing viral proteins on their surface. In the present study, we show a novel antiviral strategy based on the use of immunoliposomes containing the antimicrobial peptide melittin targeted to the glycoprotein G of the viral hemorrhagic septicemia rhabdovirus (VHSV).

2. Methods, results and discussion

Present study describes for the first time the construction of a AMP loaded-immunoliposome system targeted to a virus, such as the VHSV (one of the most devastating viruses for worldwide aquaculture (Lorenzen and LaPatra, 2005). Up to date, reports on the use of immunoliposomes as antiviral agents have been scarce. Therefore we used a similar strategy as that one previously established by Barrajon-Catalan et al. (2010) to finally obtain pegylated immunoliposomes engineered to contain covalently attached antibodies against VHSV glycoprotein G (gpG) and loaded with melittin at a lipid:peptide ratio of 1:50. Monoclonal antibodies against the extracellular domain of the gpG of VHSV were obtained from ascites of immunized mice as described before (Estepa and Coll, 1996). To eliminate the unbounded antibody and melittin, the mixture was passed through a Sephadex G-25 (Sigma–Aldrich) column using THG buffer for the elution. The presence of melittin and antibody in the immune liposomes was quantified by HPLC and SDS–PAGE gel respectively as previously described (Barrajon-Catalan et al., 2010). The average size of the immunoliposomes was also checked by using light scattering technology through intensity measurements with a Malvern ZetaSizer Nano XL (Malvern Instruments Ltd.) machine as previously described (Barrajon-Catalan et al., 2010).

The fish cell line *epithelioma papulosum cyprini* (EPC) used in this work was purchased from the European collection of cell cultures (ECACC No. 93120820). To exclude non-specific antiviral activities due to cellular toxicity, the cell viability in EPC cell monolayers was determined by the MTT assay (Mosmann, 1983) after a 24 h exposure at 14 °C to different concentrations of each liposome preparation. At 100 μM, the highest concentration of melittin loaded-liposomes and -immunoliposomes used, EPC cell monolayers showed 60.5 ± 3.8 and 66.9 ± 1.6% of viability, respectively; consequently, following studies were pursued with concentrations of 25 and 50 μM at which all treatments allowed viability rates higher than 80% (Fig. 1).

To assay for VHSV infectivity, a previously developed immunostaining focus assay was used (Lorenzo et al., 1996). To assess the influence of pre-incubation of VHSV (VHSV 07.71 isolated in France from rainbow trout (LeBerre et al., 1997) with immunoliposomes, different concentrations of the liposome constructions (25 and 50 μM) were incubated with 10^2 foci forming units (f.f.u.) from replication competent stocks of concentrated VHSV for 0, 2 and 6 h prior to infection at 14 °C in 100 μl cell culture medium supplemented with 2% serum, 2 mM L-glutamine and 50 μg/ml gentamicin. After incubation, mixtures were added to the EPC cell monolayers in 96-well plates in a final volume of 100 μl per well and incubated for 2 h at 4 °C (adsorption period). Then, the infected cell monolayers were washed and further incubated for 24 h at 14 °C until they were fixed in cold methanol, labeled with a monoclonal antibody (MAb) 2C9 directed towards the N protein of VHSV and stained with peroxidase-labelled rabbit anti-IgG mouse antibody (Nordic, Tilburg, The Netherlands) and diaminobenzidine (DAB) (Sigma). Melittin loaded-liposomes and immunoliposomes inhibited VHSV-infected cell foci formation at both concentrations used (Fig. 2, left panels). Inhibitory activity was both dose and time-dependent, thus highest inhibition rates (about 89.9% and 95.2% for melittin loaded-liposomes and immunoliposomes, respectively) were obtained with treatments using 50 μM liposomes incubated for 12 h. Melittin loaded-immunoliposomes seemed to be more active at both concentrations; however, the most significant differences between melittin loaded-liposomes and immunoliposomes were obtained at the 2 h incubation period for the concentration of 50 μM (39.5 ± 0.72 and 9.7 ± 1.9% respectively).

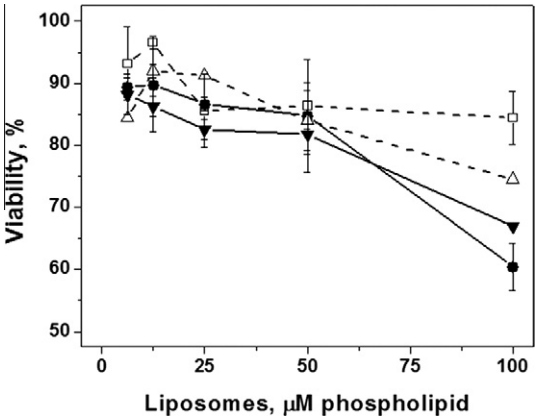

Fig. 1. Cellular toxicity of immunoliposomes. Immunoliposomes (6.25–100 μM phospholipid concentration) were added to EPC cell monolayers and assayed for cellular toxicity by the MTT viability assay after 24 h. Percentage of cellular toxicity was calculated by the formula, absorbance at 570 nm of EPC monolayers treated with immunoliposomes × 100/absorbance at 570 nm of control EPC monolayers. Data are expressed as means ± standard deviations of six determinations per point and are representative of one experiment. □, liposomes; Δ, immunoliposomes; ●, melittin loaded-liposomes; ▼, melittin loaded-immunoliposomes.

Alternatively, experiments were set to further investigate the antiviral activity of the liposome constructions after virus adsorption. For this purpose, treatments were added to EPC cell monolayers at time point 0 (just after adsorption) and 4 h post-infection, i.e. treatments were incubated with the infected cell monolayers for 24 (the full infection period) and 20 h, respectively. The results showed that melittin loaded-liposomes and immunoliposomes were also able to inhibit the infectivity of VHSV even after the adsorption step (Fig. 2, right panels). As expected, highest inhibitory values were obtained at 0 h post-infection for treatments at 50 μM (about 55.0 and 64.1% for melittin loaded-liposomes and immunoliposomes, respectively). Again, melittin loaded-immunoliposomes treatment seemed to be slightly more active at both concentrations (Fig. 2).

The analysis of infection foci size (number of VHSV-infected cells per infection foci) also confirmed preliminary visual observations. Thus, the results of EPC cell monolayers infected with VHSV and treated after the adsorption step with 50 μM melittin loaded-liposomes or -immunoliposomes showed not only a reduction in number of VHSV-infected cell foci but also a reduction in number of VHSV-infected cells per foci. After 24 h post-infection more than 75% of the VHSV-infected cell foci contained more than 15 cells per foci in control EPC cell monolayers (Fig. 3A, stripped bar). In con-