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Potential of mZD7349-conjugated PLGA nanoparticles for selective targeting of vascular cell-adhesion molecule-1 in inflamed endothelium

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ABSTRACT

Early diagnosis and restoring normal function of dysfunctional endothelium is an attractive strategy for prevention of inflammatory diseases such as atherosclerosis. Inhibition of cell adhesion in the process of atherosclerosis plaque formation, mediated by peptide antagonists of very late antigen-4 (VLA-4) has already been developed and evaluated both in vitro and in vivo.

In this study, for the first time, modified ZD7349 (mZD7349) peptide, as an antagonist for VLA-4, was used for targeting fluorescein isothiocyanate-loaded poly (DL-lactic-co-glycolic acid) nanoparticles (FITC-PLGA NPs). Rate of binding and internalization of mZD7349-NPs to activated human umbilical vein endothelial cells (HUVECs) were compared with that of untargeted. Effects of temperature reduction and clathrin-mediated endocytosis inhibitor (0.45 M sucrose) were also studied on the binding and internalization of mZD7349-NPs and NPs.

Results showed that binding of the conjugated NPs could be significantly blocked by pre-incubating cells with the free peptide, suggesting that the binding of NPs is mediated by attaching the surface peptide to VCAM-1 on HUVECs. Also, conjugated FITC-loaded NPs were shown to be rapidly endocytosized to a greater extent than the unconjugated ones. The binding and internalization of mZD7349-NPs and NPs were slowed down at low temperature and in the presence of sucrose with greater reductions for mZD7349-NPs.

To conclude, the peptide-NPs targeting the VCAM-1 is suggested as a theranostic carrier for lesions upregulating VCAM-1.

Introduction

Atherosclerosis as a chronic inflammatory disease is one of main causes of death in the world (Pittet and Swirski, 2011). A critical and initial step of inflammatory diseases such as atherosclerosis is activation of endothelial cells with expression of leukocyte adhesion molecules and chemokines (Davignon and Ganz, 2004). Early detection and rapid regeneration of damaged endothelial cells is expected to have a very important role in preventing atherosclerosis and its adverse events such as myocardial infarction (Rani and Paliwa, 2014). Due to limited accessibility of endothelium for imaging and pharmacological agents, discovery of sensitive and accurate strategies for targeted delivery of these agents is essential (Muzykantov, 2013).

Expression pattern of many markers or receptors such as vascular cell-adhesion molecule 1 (VCAM-1), intercellular adhesion molecule 1 (ICAM-1) and E-selectin on endothelial cells in physiological and pathological conditions are different. This difference can be used in targeted drug delivery and imaging (Iiyama et al., 1999; Chacko et al., 2011). VCAM-1, for instance, is a receptor of integrin very late antigen-4 (VLA-4). VLA-4 is expressed on most human blood cells such as monocytes. Interaction of VCAM-1 with VLA-4 plays an important role in leukocyte recruitment and development of inflammation (Iiyama et al., 1999). VCAM-1 as a leukocyte adhesion molecule has important role in initiation of molecular and cellular changes that eventually leads to inflammatory diseases such as atherosclerosis (Ley and Huo, 2001). Its features such as induction in the early stages of inflammation, exclusive expression in atherosclerotic lesions, and accessible position in proximity to the blood pool, have made it an attractive biomarker for identifying initiation of endothelial dysfunctions (Nahrendorf et al., 2009). Level

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of VCAM-1 expression on the surface of activated endothelial cells is significantly different from that of normal endothelial cells, which can be used in targeted drug delivery.

There are several reports that indicate nanocarriers which are directed against VCAM-1 are suitable for therapeutic applications in addition to diagnostic applications. Cyclopentenone prostaglandin-loaded liposomes directed against VCAM-1 by antibody were efficient for reversing atherosclerotic lesions (Homem de Bittencourt et al., 2007). Deosarkar et al. studied selectivity of polymeric particles that was conjugated by anti-VCAM-1 to sites of atherosclerosis in ApoE$^{-/-}$ mouse aorta. Their results demonstrated that these particles adhered selectively to sites of plaques (Deosarkar et al., 2008). In a report, particles against VCAM-1 showed selective and focal adhesion to sites of colonic inflammation in dextran sulfate sodium-induced murine model of colitis (Sakhalkar et al., 2003a). Kang et al. used anti-VCAM-1-Fab'-conjugated liposomes for targeted delivery of celecoxib as a poorly water-soluble drug (Kang et al., 2011).

In recent years, peptides due to their lower price, more stability and higher affinity to their receptors, have become the center of attention in targeting of NPs (Friedman et al., 2013). Peptide-conjugated magnetic NPs against VCAM-1 were used for imaging endothelial cells in atherosclerotic plaques. Anti-VCAM-1 peptide showed 12-fold higher binding affinity to VCAM-1 than to anti-VCAM-1 antibody in vitro (Kelly et al., 2005; Nahrendorf et al., 2006). Previous studies reported cyclic peptides with Leu-Asp-Val binding motif to VCAM-1 to inhibit interaction of VLA-4 with VCAM-1 (Dutta et al., 2000).

In this work, for the first time, we used a cyclic peptide with high inhibitory activity (sequence cyclo (MePhe-Leu-Asp-Val-D-Arg-D-Arg, ZD7349) for targeting drug delivery (Dutta et al., 2000). Anti-inflammatory effects of this peptide have been investigated in animal models of inflammatory diseases (Dutta et al., 2000), while its effectiveness in targeting of drug carriers has not been reported yet. VCAM-1 was chosen as the target receptor on active human umbilical cord vascular endothelial cells (HUVECs). Modified ZD7349 (mZD7349) with sequence cyclo (MePhe-Leu-Asp-Val-D-Arg-D-Lys) was conjugated to surface of PLGA NPs for active targeting of activated HUVECs. We studied internalization rate of mZD7349-FITC-PLGA NPs (mZD7349-NPs) in comparison with unconjugated ones (NPs). Clathrin-mediated endocytosis is the main pathway of ligated VCAM-1 internalization onto endothelial cells (ECs). This pathway is an energy-dependent pathway and is blocked by sucrose and chlorpromazine due to inhibition of clathrin-coated pit formation (Ricard et al., 1998). In this study, we investigated effects of temperature reduction and a clathrin-mediated endocytosis inhibitor (i.e. 0.45 M sucrose) on the binding and internalization of mZD7349-NPs and NPs. To the best of our knowledge, this is the first report for selective targeting of dysfunctional endothelial cells using mZD7349-NPs, directed against VCAM-1.

Materials and methods

Materials

PLGA (50:50, Mw 50,000 g · mol^{-1}) was from Shenzhen Esun Industrial Co. (China). 1-Ethyl-3-[3-dimethylaminopropyl] carbodiimide hydrochloride (EDC), N-hydroxysuccinimide (NHS), cell dissociation solution, sucrose, Dodecyltrimethylammonium bromide (DTAB), 3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide (MTT), 4′, 6-diamidino-2-phenylindole (DAPI), Fluorescein isothiocyanate (FITC) and 2-(*N*-morpholino) ethanesulfonic acid (MES) were purchased from Sigma-Aldrich (USA). Dulbeccos modified eagles medium (DMEM), fetal bovine serum (FBS), Phosphate-buffered saline (PBS, 0.01 M, pH 7.4), penicillin and streptomycin antibiotic mixture were purchased from life technologies (USA). Acetone (99.9%, HPLC grade) was prepared from Merck chemicals (Germany). Mouse IgG1 [ICIG1] (phycoerythrin) – Isotype control (ab91357) and mouse mAb to VCAM-1 (10G11B1) (phycoerythrin) (ab82438) were purchased from Abcam (USA). HUVECs (at passages 4 to 6) were obtained from Pasteur Institute, Tehran, Iran.

Methods

Synthesis of modified Z-peptide

mZD7349 peptide was synthesized by solid phase peptide synthesis methods using 2-chlorotritylchloride resin and various coupling and blocking reagents with some modifications as described by Dutta et al. (2000). Second D-Arg was replaced with D-Lys. Structure and sequence of synthesized peptide was confirmed by amino acid analysis and mass spectroscopy.

VCAM-1 expression by TNF-α

Flow cytometry was used in determination of VCAM-1 expression for activated HUVECs. HUVECs were grown in DMEM medium supplemented with 10% FBS, 0.1% non-essential amino acids, and 0.1% penicillin–streptomycin at 37 °C in a humidified 5% CO_2 atmosphere. HUVECs with 90% confluence in a six-well plate (5×10^5 cells/well) were treated with TNF-α (10 ng/ml) for 6 h at 37 °C. The cells were detached by cell dissociation solution (incubated for 20 min, at 37 °C) and pipetting. Then, the cells were washed with PBS by centrifugation (800 g, 5 min at 4 °C) and resuspended in 50 μl PBS containing mouse monoclonal antibody (phycoerythrin-conjugated primary antibody) against VCAM-1. Also, cells incubated by antibody against IgG-1 were used as isotype control. After reacting for 30 min at room temperature, cells were washed twice with PBS by centrifugation (800 g, 5 min at 4 °C). Cells were then fixed in 1% paraformaldehyde for 5 min at 20 °C and stored at 4 °C to analysis time. Cells which were not labeled by antibody were used as control. Flow cytometry was performed by fluorescence activated cell scan (FACS) analyzer (Becton Dickinson; Mountain View, CA) at excitation and emission wavelength values of 488 and 575 nm, respectively, using CellQuest software (BD Biosciences).

Confirmation of binding of peptide to VCAM-1

Flow cytometry was performed for confirming binding of synthesized peptide (mZD7349) as an inhibitor of interaction between anti-VCAM-1 and VCAM-1. HUVECs with 90% confluence in a six-well plate (5×10^5 cells/well) were activated with TNF-α (10 ng/ml) for 6 h at 37 °C. The cells were washed three times with PBS (pH 7.4) and incubated by mZD7349 (0.5 mg/ml) for 30 min at 37 °C. Untreated cells were used as control. Cells were again washed with PBS and remaining steps were performed similar to the procedure described in 2-2-2.

Preparation of FITC-loaded PLGA nanoparticles

FITC as a fluorescent marker was loaded onto PLGA NPs using double-nozzle electrospraying. The spraying apparatus consisted of a high-voltage electrical power supply, a mechanical syringe pump and two stainless steel nozzles with an inner and outer diameter of 1.77 and 2.34 mm, respectively. In brief, FITC (0.07% *w*/*v*), DTAB salt (0.8 mM) and PLGA (0.7% *w*/*v*) were dissolved in acetone by sonication. Subsequently, the solution was sprayed using the pump.

Conjugation of mZD7349 peptide to NPs

EDC and NHS were used as coupling agents for covalent coupling of carboxyl terminates of NPs. Fifteen milligram of NPs were dispersed in MES buffer (0.05 M, pH 5.4). NPs were then incubated for 60 min with 24 mM EDC and 6 mM NHS. Covalent coupling of the NHS-activated particles with the amino groups of the peptide (0.5 mg/ml) was performed for 4 h, at room temperature. Size, size distribution, and zeta potential (pH 7.4) of NPs and mZD7349-NPs were evaluated using Zetasizer (Malvern, UK). Additionally, fluorescent intensity of released FITC molecules from both mZD7349-NPs and NPs (300 μg/ml) were studied using fluorometry at 30, 60 and 120 min in serum free medium without cell.