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Note

Peptide-targeted PEG-liposomes in anti-angiogenic therapy

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Abstract

Peptides with the RGD amino acid sequence show affinity for the alpha(v)beta(3) integrin, an integrin which is over-expressed on angiogenic endothelium and involved in cell adhesion. A peptide with the sequence ATWLPPR has been demonstrated to show affinity for the vascular endothelial growth factor (VEGF) receptor, a receptor involved in the proliferation of endothelial cells. By coupling these peptides to liposomes, these liposomes can serve as a site-specific drug delivery system to tumor endothelial cells in order to inhibit angiogenesis. In the present study we demonstrate that the coupling of cyclic RGD-peptides or ATWLPPR-peptides to the surface of PEG-liposomes results in binding of these liposomes to endothelial cells in vitro. Subsequent studies with RGD-peptide targeted liposomes in vivo also demonstrate specific binding to the tumor endothelium.

Keywords: Angiogenesis; RGD-peptides; Liposomes; Endothelial cells

The formation of new blood vessels out of pre-existing ones, also known as angiogenesis, is considered to be essential for tumors to grow beyond a size of 2–3 mm^3 (Griffioen and Molema, 2000). Therefore, inhibition of angiogenesis is an attractive method to reduce tumor growth. In contrast to the normal quiescent endothelium, the activated tumor endothelial cells over-express certain surface markers, involved in angiogenesis. These surface markers discriminate tumor endothelial cells from the normal endothelial cells and can be used as a target for anti-angiogenic therapy. One group of surface markers are the integrins. Integrins are trans-membrane proteins consisting of an alpha and a beta subunit and are involved in the migration, proliferation and maturation of endothelial cells. The alpha(v)beta(3) integrin mediates cellular adhesion to vitronectin, fibronectin, fibrinogen, thrombospondin, proteolyzed collagen, von Willebrand factor (vWF) and osteopontin by recognizing a so-called RGD (Arg–Gly–Asp) sequence (Ruoslahti, 1996). Peptides containing an RGD sequence have been developed as ligands with a high affinity for these integrins. RGD-peptides and LM609 (an anti-alpha(v)beta(3) integrin mAb) have shown to prevent tumor growth in vivo by disruption of the alpha(v)beta(3) ligation (Brooks et al., 1994; Drake et al., 1995; Eliceiri and Cheresh, 1999).

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Blocking the interaction between vascular endothelial growth factor (VEGF) and the VEGF-receptor (KDR/FLK1 or VEGFR-2) is another promising method to inhibit angiogenesis. A peptide with the sequence ATWLPPR has been shown to be an effective antagonist of VEGF and has affinity for the VEGF-receptor, which is over-expressed on the surface of angiogenic endothelial cells (Binetruy-Tournaire et al., 2000).

In the present study, the RGD- or ATWLPPR-peptides were coupled to PEG-liposomes to allow multivalent display of the binding peptides with the objective of site-specific drug delivery to tumor endothelium.

Preparation of PEG-liposomes

Liposomes were prepared as described previously (Vingerhoeds et al., 1996). In brief, a lipid film was made from dipalmitoylphosphatidylcholine, cholesterol, PEG–distearoylphosphatidylethanolamine (DSPE) and maleimide–PEG–DSPE in a molar ratio of 1.85:1.0:0.075:0.075, respectively. For fluorescent labeling of the liposomes 0.1 mol% 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindodicarbocyanine perchlorate (DiD) or lissamine-rhodaminephosphatidylethanolamine (Rho-PE) was added. PEG-liposomes were formed by addition of Hepes NaCl-buffer pH 6.7 to the lipid film. Liposome size was reduced by multiple extrusion steps through polycarbonate membranes to 100 nm.

Coupling of the peptide

The linear ATWLPPR and cyclic 5-mer RGD- and RAD-peptides were synthesized with C-terminal thio-acetyl group, at a purity of 95%. The RAD-peptide was used as a non-binding control peptide (Ruoslahti, 1996). Appropriate amounts of acetyl protected-peptide were deacetylated in an aqueous solution containing 0.5 M Hepes/0.5 M hydroxylamine–HCl/0.25 mM ethylenediaminetetraacetic acid of pH 7.0 for 30 min at room temperature. Subsequently, the activated peptide was incubated with the liposomes to form a thio-ether bond with the maleimide–PEG–DSPE-incorporated in the lipid bilayer, based on a reaction described by Vingerhoeds et al. More than 99% of the peptide reacted with the maleimide–PEG–DSPE. The hydroxylamine solution was removed by gel chromatography.

Cells

Human umbilical vein endothelial cells (HUVEC) were cultured in RPMI 1640 medium containing 25 mM Hepes and 2 mM L-glutamine supplemented with 20% (v/v) heat-inactivated fetal calf serum and antimicrobial agents. HUVEC were used up to passage 3.

FACS analysis of liposome binding to HUVEC

HUVEC growing in a confluent monolayer were detached using EDTA and suspended in FACS-buffer (PBS supplemented with 1% bovine serum albumin (BSA), $CaCl_2$ (1.26 mM) and $MgSO_4$ (0.81 mM). Cells were incubated with the DiD labeled PEG-liposomes (PEG-L), RAD-peptide–PEG-liposomes (RAD–PEG-L), RGD-peptide–PEG-liposomes (RGD–PEG-L) or ATWLPPR-peptide–PEG-liposomes for 1 h at 4 °C. In the dissociation experiment, RGD–PEG-L were allowed to associate to the HUVEC for 30 min at 4 °C, subsequently increasing concentrations of either free RAD or free RGD-peptide were added and incubated for another 30 min at 4 °C. Cells were analyzed by flow cytometry using a FACScalibur (Becton-Dickinson). Results were analyzed using WinMDI software, version 2.8 (Joseph Trotter, USA).

Confocal laser scanning microscopy (CLSM) analysis of liposome handling by HUVEC

HUVEC were detached and transferred to fibronectin-coated 12 well-chamber slides, allowed to adhere overnight and subsequently incubated with Rho-PE labeled PEG-L, RAD–PEG-L or RGD–PEG-L. After incubation, cells were washed with PBS (4 °C), fixed in 2% buffered formaldehyde and stored overnight at 4 °C. Next, plastic well separations were removed and the slide was covered in glycerin:kaisers glyceringelatin (1:1, v/v) and 2.5% 1,4-diazabicyclo-(2,2,2)-octane. The samples were then examined by CLSM using a Leica TCS-SP spectral confocal microscope.

Visualization of tumor vasculature binding of liposomes by intravital microscopy

The preparation of the dorsal skin-fold chamber is an adaptation from the previously described

procedure by Papenfuss et al. (1979). A small piece of Lewis lung carcinoma (0.1 mm^3) was transplanted in the fascia using a micro-surgical microscope. After implantation of the tumor tissue the mice were housed in an incubation room with an ambient temperature of 35 °C and a humidity of 50%. The tumor was allowed to reach an approximate size of 5 mm^3.

Mice bearing the skin-fold chamber were anesthetized and fixed to a microscope stage. Next, PEG-liposomes were injected intravenously. Observations of the tumor vasculature were made with a Leica DM-RXA fluorescence microscope. Images of the tumor were acquired using a Sony 3CCD DXC950 digital color video camera connected to a PC. Image acquisition and image analysis was performed with Research Assistant 3.0 for Windows 98.

FACS analysis of liposome binding to HUVEC

HUVEC were incubated with increasing concentrations of DiD labeled PEG-L, RAD–PEG-L or RGD–PEG-L. The RGD–PEG-L showed a six-fold increase in binding to HUVEC than PEG-L and RAD–PEG-L over the entire concentration range. ATWLPPR–PEG-liposomes displayed a 10-fold higher binding as compared to PEG-L.

To demonstrate the specificity of binding of the RGD–PEG-L, HUVEC were incubated with DiD labeled RGD–PEG-L followed by incubation with increasing concentrations of free RGD or RAD-peptide. Only the free RGD-peptide was able to dissociate the RGD–PEG-L. In this way, about 50% of the bound RGD–PEG-L could be displaced. The free RAD-peptide was not able to dissociate the bound RGD–PEG-L, despite the use of high concentrations of free peptide.

CLSM analysis of liposome binding to and internalization by HUVEC

Liposome processing was analyzed by incubating HUVEC with Rho-PE labeled PEG-L or RGD–PEG-L for 1 and 3 h at 37 °C. After 1 h incubation fluorescence was prominently visible after incubation with RGD–PEG-L, this in contrast to PEG-L. After 3 h incubation the interaction of RGD–PEG-L was even more pronounced.

Visualization of tumor vasculature binding of liposomes by intravital microscopy

To demonstrate adhesion of RGD–PEG-L to vascular tumor endothelium, liposomes were injected intravenously and visualized using intravital microscopy. Already at 2 min after administration small clusters of fluorescence were observed inside the tumor blood vessels. Within 30 min large clusters of fluorescence were seen to adhere to the vessel wall. In contrast, after injection of the PEG-L and RAD–PEG-L no clustering of fluorescence inside the vessels was visible. Instead, fluorescence was observed outside the vessels, which did not occur in case of RGD–PEG-L. This suggests that PEG-L or RAD–PEG-L extravasate deeper into the tumor interstitium.

The RGD–PEG-liposomes demonstrate a higher affinity binding to tumor endothelial cells in vitro than RAD–PEG-liposomes or non-targeted PEG-liposomes. ATWLPPR–PEG-liposomes also exhibited increased affinity for endothelial cells in vitro. In vivo studies with the RGD–PEG-liposomes showed that targeting of proliferating endothelial cells surrounding tumors also occurred in vivo. Future studies will focus on the incorporation of a therapeutic drug in RGD–PEG-liposomes to reduce tumors.

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