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Light-enhanced $VEGF_{121}$/rGel: A tumor targeted modality with vascular and immune-mediated efficacy

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ABSTRACT

Interactions between stromal cells and tumor cells pay a major role in cancer growth and progression. This is reflected in the composition of anticancer drugs which includes compounds directed towards the immune system and tumor-vasculature in addition to drugs aimed at the cancer cells themselves. Drug-based treatment regimens are currently designed to include compounds targeting the tumor stroma in addition to the cancer cells. Treatment limiting adverse effects remains, however, one of the major challenges for drug-based therapy and novel tolerable treatment modalities with diverse high efficacy on both tumor cells and stroma is therefore of high interest. It was hypothesized that the vascular targeted fusion toxin $VEGF_{121}$/rGel in combination with the intracellular drug delivery technology photochemical internalization (PCI) stimulate direct cancer parenchymal cell death in addition to inhibition of tumor perfusion, and that an immune mediated response is relevant for treatment outcome. The aim of the present study was therefore to elucidate the anticancer mechanisms of $VEGF_{121}$/rGel-PCI. In contrast to $VEGF_{121}$/rGel monotherapy, $VEGF_{121}$/rGel-PCI was found to mediate its effect through VEGFR1 and VEGFR2, and a targeted treatment effect was shown on two VEGFR1 expressing cancer cell lines. A cancer parenchymal treatment effect was further indicated on H&E stains of CT26-CL25 and 4 T1 tumors. $VEGF_{121}$/rGel-PCI was shown, by dynamic contrast enhanced MRI, to induce a sustained inhibition of tumor perfusion in both tumor models. A 50% complete remission (CR) of CT26.CL25 colon carcinoma allografts was found in immunocompetent mice while no CR was detected in CT26.CL25 bearing athymic mice. In conclusion, the present report indicate $VEGF_{121}$/rGel –PCI as a treatment modality with multimodal tumor targeted efficacy that should be further developed towards clinical utilization.

1. Introduction

Vascular targeted therapeutics have, in recent years, emerged as adjuvants to standard anticancer therapeutics. Most of the clinically approved vascular targeted agents are anti-angiogenic drugs such as bevacizumab, sunitinib and sorafenib, which act on VEGF receptor (VEGFR) signal transduction and inhibit the formation of new blood vessels [1,2]. Tumor blood supply may, however, also be targeted through vascular disruptive agents, a broad class of compounds with the potential to damage already established vasculature [3,4]. $VEGF_{121}$/rGel is a vascular disrupting agent consisting of the type I ribosome inactivating protein toxin gelonin and $VEGF_{121}$, an isomer of VEGF-A [5]. $VEGF_{121}$/rGel is a hydrophilic macromolecule which is taken up by the cells through VEGFR1- and VEGFR2- receptor-mediated endocytosis. Repeated systemic administration of $VEGF_{121}$/rGel induces a significant tumor growth delay in several animal models and has also been shown to reduce the formation of bone metastasis in a prostate tumor model in mice [6,7]. Gelonin lacks an effective transport mechanism to enter the cell cytosol upon endocytosis [8,9], and it has been hypothesized that the cytotoxic effect of gelonin-based therapeutics is caused by only a small fraction of the administrated dose while the majority of the drug is subjected to lysosomal degradation

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[10]. This has indeed been demonstrated by the enhanced therapeutic effect of gelonin-based targeted toxins when combined with photochemical internalization (PCI), a modality for cytosolic release of drugs entrapped in endo/lysosomal compartments [11–15]. PCI is based on amphiphilic photosensitizers, such as $TPPS_{2a}$ and $TPCS_{2a}$ [16], which accumulate in the membranes of endo/lysosomal vesicles [17,18]. Light exposure at the appropriate wavelength activates the photosensitizer and induce the formation of reactive oxygen species (ROS) that destabilize the endo/lysosomal membrane. Compared to other available modalities for endosomal escape, such as chloroquine, saponin and proton sponge, PCI should have an advantage due to cancer selectivity. The photosensitizer is retarded 2–3 fold in tumor tissue compared to normal tissue, and the light exposure is confirmed to the tumor area [16]. This advantage should provide superior tumor targeting properties when used in combination with a tumor-targeted drug such as $VEGF_{121}$/rGel. PCI has already gone through a clinical phase I/II study where it was found tolerable and highly efficient [19]. This is in contrast to choloquine, as well as other modalities for endosomal escape, which have turned out to toxic for evaluation in preclinical models [20,21].

PCI has mostly been utilized for the delivery of hydrophilic macromolecular drugs to cancer cells [18]. Endothelial cells have, however, also been identified as a promising target for PCI induced drug delivery [22]. $VEGF_{121}$/rGel-PCI has been reported as specific for VEGFR2 expressing endothelial cells compared to the tumor cell line CT26.CL25. In addition, immunohistochemistry (IHC) of $VEGF_{121}$/rGel-PCI treated tumors has demonstrated a collapse of CD31 staining vessels [14]. There is a general assumption that targeting of the tumor vascular system usually is not sufficient to obtain a complete remission (CR) of cancer, and vascular targeted monotherapy is seldom used in a curative setting [2]. The high response rate (40% CR) following $VEGF_{121}$/rGel-PCI in the highly aggressive CT26.CL25 model has therefore been reported as surprising and highly promising [14]. The present study was designed to elucidate the mechanisms resulting in the high efficacy of $VEGF_{121}$/rGel-PCI. We here show that the overall treatment effect of $VEGF_{121}$/rGel-PCI is not only dependent on a vascular response, but most likely also is a result of tumor cell targeting mediated through VEGFR1. The direct effect on the tumor cells my impact on the indicated immune-mediated response which seems essential for treatment induced remission.

2. Materials and methods

2.1. Cell lines and culture conditions

The native, VEGFR1-transfected and VEGFR2-transfected porcine aortic endothelial cell lines (PAE/control, PAE/VEGFR1 and PAE/VEGFR2) were provided by Dr. Johannes Waltenberger. CT26.CL25, CT26 and 4 T1 murine cancer cells were obtained from ATCC (Manassas, VA, USA). The PAE cell lines were maintained in Nutrition F-12 Ham medium (Sigma Aldrich, St Louis, MO) supplied with 10% FCS, 100 U/ml penicillin and 100 μg/ml streptomycin. CT26.CL25 was maintained in RPMI 1640 (Sigma Aldrich) supplied with 0.23% D (+) Glucose and 1 mM sodium pyruvate in addition to FCS and penicillin/streptomycin. 4 T1 and CT26 cells were maintained in RPMI medium supplied with FCS and penicillin/streptomycin. All cell lines were used between passage numbers 1–25 and routinely checked for *Mycoplasma* sp. infections.

2.2. Photochemical internalization of $VEGF_{121}$/rGel in vitro

PAE/control, PAE/VEGFR1, PAE/VEGFR2, CT26.CL25 and 4 T1 cells were seeded at 3000 cells/well in 96 well plates for 5 h, followed by incubation with $TPPS_{2a}$ (PCI Biotech, Oslo Norway) for 18 h. The cells were washed twice with PBS and incubated for 3 h in drug free medium before they were subjected to a 1 h incubation with $VEGF_{121}$/rGel (recombinantly produced as previously described [5]) or gelonin [23]. The cells were then washed once with medium before they were illuminated with LumiSource (PCI Biotech) emitting blue light with a λ_{max} = 435 nm. Viability was assessed by the MTT method [24] at indicated time points. VEGFR selectivity was evaluated by co-incubation of 0.1 nM $VEGF_{121}$/rGel and 10 μM Bevacizumab (Avastin, Roche), or by adding 0.5 μM Sunitinib (Sigma Aldrich) from 3 h prior to until 2 h after light exposure (sunitinib was replaced directly after light expsoure in case of photochemical damage [25]). Impact of cellular cholesterol was assessed by incubating cells with 5 μM methyl-β-cyclodextrine for 3 h after removing $TPPS_{2a}$ followed by co-incubating $VEGF_{121}$/rGel with 5 μM methyl-β-cyclodextrine for 1 h prior to washing and light exposure.

2.3. Cellular uptake of $VEGF_{121}$/rGel

In addition to confocal microscopy of Alexa Fluor 488-$VEGF_{121}$/rGel, intracellular accumulation of $VEGF_{121}$/rGel was visualized by Western blot analysis of $VEGF_{121}$/rGel-PCI treated PAE/VEGFR1 and PAE/VEGFR2 cell lysates using anti-gelonin- [5] and anti-actin (#A5060, Sigma Aldrich) antibodies. The sample preparation, SDS-PAGE and Western blotting were performed as previously described [13].

2.4. Cell fractionation and assessment of cholesterol

Subcellular fractionation with centrifugation was done as previously reported [26]. The plasma membrane was broken using a hypotonic buffer in combination with mechanical stress followed by an isotoic adjustment to avoid damage to the endo/lysosomal membranes. Briefly, a 50% confluent 75 cm^2 flask of cells was trypsinized and centrifuged at 235 × *g* for 4 min and resuspended in 1 ml PBS. The sample was divided in 2 × 500 μl fractions, one representing whole cells and one which was subjected to further fractionation as follows: (i) Centrifugation for 4 min at 235 × *g*, resuspension of the pellet in a hypotonic buffer (10 mM HEPES, 1.5 mM $MgCl_2$, 10 mM KCl pH 7.4) followed by a 30 min incubation at 4 °C. The cells were then sheared through a 23 G needle to destroy the plasma membrane. 27 μl NaCl was then added to obtain an isotonic solution. (ii) The samples were further subjected to a 10 min centrifugation at 800 × *g* at 4 °C to isolate the nucleus (pellet). The supernatant was further centrifuged for 20 min at 28,000 × *g* at 4 °C to isolate lysosomes and endosomes (pellet). The cytoplasm was defined as the final supernatant. The amount of cholesterol in each sample was assessed by the Amplex® Red Cholesterol Assay Kit (Thermo Fisher, Waltham, MA) according to the manufacturer's instructions and calculated relative to the amount of proteins in each sample as assessed by the DC™ Protein Assay (Biorad, Hercules, CA).

2.5. Confocal microscopy

$VEGF_{121}$/rGel was labeled with Alexa Fluor 488 using the Alexa Fluor 488 Protein Labeling Kit (Thermo Fisher). Prior to microscopy cells were seeded at 200,000 cells/well in 6 well plates with cover slips (Assistant, Sondheim, Germany, No. 1001/10). The cells were attached overnight and incubated with 100 nM Alexa 488-$VEGF_{121}$/rGel for 1 h prior to PFA fixation on ice for 15 min. Cells were washed with PBS containing 0.05% saponin for 2 × 10 min before they were subjected to antibody incubation in a humidified chamber with the primary antibodies targeting early endosome antigen 1(αEEA1) (#610456, Transduction Laboratories, Lexington, KY) and Lysosomal-associated membrane protein (αLAMP1) (#L1418, Sigma Aldrich) and the secondary antibodies α-mouse-Alexa 555 (Molecular Probes) and α-rabbit-Alexa 647 (Jackson Immunoresearch, Suffolk, UK). The cover slips were mounted using Mowiol mounting media (Sigma Aldrich) containing Hoechst. Confocal microscopy was performed using a confocal microscope (Carl Zeiss MicroImaging GmbH, Jena, Germany) as previously