Journal of Controlled Release 251 (2017) 1–10

Contents lists available at ScienceDirect

Journal of Controlled Release

journal homepage: www.elsevier.com/locate/jconrel

Modality of tumor endothelial VEGFR2 silencing-mediated improvement in intratumoral distribution of lipid nanoparticles

Shoshiro Yamamoto [1], Akari Kato [1], Yu Sakurai [1], Tomoya Hada, Hideyoshi Harashima *

Faculty of Pharmaceutical Sciences, Hokkaido University, Japan

ARTICLE INFO

Article history:
Received 8 December 2016
Received in revised form 16 January 2017
Accepted 8 February 2017
Available online 10 February 2017

Keywords:
Liposome
Intratumoral distribution
Tumor microenvironment
siRNA
Tumor vasculature

ABSTRACT

The vascular endothelial growth factor (VEGF)-mediated enhancement in vascular permeability is considered to be a major factor in tumor-targeting delivery via the enhanced permeability and retention (EPR) effect. We previously reported that the silencing of the endothelial VEGF receptor (VEGFR2) by a liposomal siRNA system (RGD-MEND) resulted in an enhanced intratumoral distribution of polyethylene glycol (PEG)-modified liposomes (LPs) in a renal cell carcinoma, a type of hypervascularized cancer, although the inhibition of VEGF signaling would be expected to decrease the permeability of the tumor vasculature. We herein report that the enhancement in the intratumoral distribution of LPs by VEGFR2 inhibition was dependent on the vascular type of the tumor (stroma vessel type; SV and tumor vessel type; TV). In the case of TV-type tumors (renal cell carcinoma and hepatocellular carcinoma), inhibiting VEGFR2 improved intratumoral distribution, while no effect was found in the case of SV-type tumors (colorectal cancer). Moreover, through a comparison of the intratumoral distribution of LPs with a variety of physical properties (100 nm vs 400 nm, neutral vs negative vs positive), VEGFR2 inhibition was found to alter the tumor microenvironment, including heparan sulfate proteoglycans (HSPGs). In addition, the results regarding the effect of the size of nanoparticles indicated that VEGFR2 inhibition improved the penetration of nanoparticles through the vessel wall, but not via permeability, suggesting the involvement of an unknown mechanism. Our findings suggest that a combination of anti-angiogenic therapy and delivery via the EPR effect would be useful in certain cases, and that altering the tumor microenvironment by VEGFR2 blockade has a drastic effect on the intratumoral distribution of nanoparticles.

1. Introduction

The enhanced permeability and retention (EPR) effect, a major strategy for delivering macromolecules to tumor tissue, is characterized by "1) hypervasculature, 2) enhanced vascular permeability as elicited by a factor, 3) negligible recovery of macromolecules via the blood vessels and 4) negligible recovery from the lymphatic system", as reported in the first study by Maeda dealing with the EPR effect [1]. Since this phenomenon was reported, a number of nanodrug delivery systems (DDSs) based on the EPR effect have been developed [2]. Enhanced vascular permeability, which is driven by the vascular endothelial growth factor (VEGF) produced by various cells in tumor tissue, such as cancer cells, macrophages [3], fibroblasts [4], is considered to be a dominant factor in EPR effect-based delivery [5]. Actually, several enhancers of permeability, including VEGF itself, nitric oxide (NO) and bradykinin, have been reported to improve the therapeutic efficacy of EPR effect-based nanoparticles [6]. Thus, the immature, permeable vasculature in the tumor tissue is believed to mainly contribute to the extravasation of nanoparticles from the tumor vasculature to the extracellular space.

In contrast, we recently reported that the siRNA-mediated silencing of endothelial VEGF receptor 2 (VEGFR2) induced the maturation of the tumor vasculature and an unexpected elevation in tumor accumulation and the penetration of nanoparticles in a renal cell carcinoma (RCC) model of hypervascularized cancer [7]. These results were inconsistent with previously reported findings in which permeability enhancers were reported to improve EPR effect-based delivery. We focused on remodeling the extracellular matrix (ECM) via VEGFR2 inhibition because the degradation of collagen and hyaluronic acid to low molecular weight compounds by enzymes also enhanced the EPR-based delivery of nanoparticles [8–10]. As a result, we found that VEGFR2 inhibition via siRNA delivery increased the numbers of infiltrating M1 type macrophages, and subsequently collagen degradation by matrix metalloproteinases (MMPs) produced from the increased numbers of macrophages, thus leading to an improved EPR effect-based delivery of nanoparticles in hypervascularized RCC. This discovery suggests that permeability is not the only determinant factor of the efficacy of EPR effect-based delivery. In other words, it would be wise to take into account the entire tumor microenvironment, including the number of cancer cells, other cell populations (macrophages, fibroblast, etc.),

* Corresponding author at: Kita-12, Nishi-6, Kita-ku, Sapporo 060-0812, Japan.
E-mail address: harasima@pharm.hokudai.ac.jp (H. Harashima).
[1] These three authors equally contributed to this manuscript.

http://dx.doi.org/10.1016/j.jconrel.2017.02.010
0168-3659/

ECMs, blood flow, to achieve the maximum outcome in the case of nanoparticle-based delivery. For purposes of this study, we defined the term "tumor accumulation" as the number of nanoparticles that flowed in the effective bloodstream, and subsequently penetrated tissue via the vessel wall (Fig. S1). This was distinguished from the term "intratumoral distribution", which is defined as the extent of nanoparticles that diffused from the vasculature against the steric hindrance from cells and ECMs (Fig. S1).

The use of anti-angiogenic therapy for cancer patients, such as anti-VEGF antibody Bevacizumab, anti-VEGFR2 antibody Ramucirumab, multi-kinase inhibitors (Sunitinib, Sorafenib, etc.) and inhibitors against mammalian target of rapamycin (mTOR) [11,12] continues to expand. Considering this present situation regarding anti-cancer treatment, the beneficial use of a combination of antiangiogenic therapy and nanoparticles should be attempted in future clinical trials. However, according to our traditional understanding of the EPR effect, anti-angiogenic therapy would be expected to reduce therapeutic outcomes by nanoparticles because VEGF inhibition reduces the permeability of the tumor vasculature [13,14]. Concerning low molecular weight compounds, anti-angiogenic therapy enhances the efficacy of therapeutics. For example, Irinotecan, Fluorouracil and Leucovorin with Bevacizumab were found to be superior to that without Bevacizumab in overall survival rate in cases of untreated metastatic colorectal cancer (CRC) patients [15]. This improvement by inhibiting VEGF signaling in the therapeutic effects of low molecular weight compounds can be attributed to a recovery of blood flow, the inhibition of leakage in outer areas of the tumor tissue via lowered permeability and a decrease in interstitial fluid pressure (IFP) [16]. On the other hand, the literature on relationships between anti-VEGF therapy and EPR effect based delivery is sparse. In a human lung cancer model, VEGFR and platelet-derived growth factor (PDGF) R inhibitor Pazopanib failed to improve the delivery of doxorubicin-loaded liposomes [17], while in case of murine colorectal cancer, SU5416, a VEGFR2 inhibitor formulated into an emulsion, enhanced the penetration of polyethylene glycol (PEG)-liposomes [18]. In another study, Chauhan et al. reported that the improvement in the delivery of nanoparticles via VEGFR2 inhibition by the antibody DC101 was dependent on the size of nanoparticles by comparing the effect with Abraxane (12 nm) and Doxil (100 nm) [19]. No improvement was found for the intratumoral distribution of large size nanoparticles (100 nm) by VEGFR2 inhibition, while that for smaller sized nanoparticles (12 nm) was. Taken together, the effect of VEGF inhibition on the intratumoral delivery of nanoparticles remains a controversial subject, suggesting that its precise effect should be clarified for continued progress in the area of EPR effect-based delivery.

The objective of this study was to elucidate the precise mechanism responsible for the intratumoral distribution of nanoparticles that occur under conditions of VEGF inhibition conditions by using different types of nanoparticles and cancer cells. In this study, we prepared clusters of cancer cells based on spatial correlation of cancer cells and stroma including vasculature, as described in a previous report [20], leading to the producing stromal vessel type (SV) and tumor vessel type (TV) clusters. This clustering was first proposed in a study by Smith et al. [20]. They classified various cancer types according to tumor stroma architecture of clinical tumor specimens; namely, vessels that were distributed around the tumor cells (TV) and vessels that were associated with stromal cells (SV). TV-type clusters were sensitive to anti-VEGF therapy while SV-type clusters did not respond to anti-angiogenic treatment. Therefore, they concluded that this structural phenotype determined the extent of response to anti-VEGF therapy. We hypothesized that these vasculature phenotypes also determined the effect of VEGFR2 inhibition on the distribution of nanoparticles in the tumor tissue. In addition, to determine the optimized carrier and obtain further information on the effect of VEGF blockade, alterations in the intratumoral distribution of various types of liposomes (LPs) (neutral, anionic, cationic; small, large) were monitored.

To specifically silence endothelial VEGFR2 expression, we used a cyclic RGD peptide-modified liposomal siRNA system. We previously developed a system for delivering liposomal nucleic acids, a multi-functional envelope-type nanodevice (MEND) [21–23]. The lipid envelope of the RGD-MEND consists of a pH-sensitive cationic lipid YSK05. YSK05 exhibits fusogenic properties at an acidic pH (<5.5) [24], which allows the RGD-MEND to escape from endosomes after being internalized. On the other hand, because YSK05 is neutral at physiological pH 7.4, a YSK05-containing MEND (YSK-MEND) would have a high biocompatibility. These characteristic properties of the YSK-MEND enabled us to inhibit mRNA expression, even in in vivo situations, such as the murine liver [25–28], a humanized chimeric liver infected with human hepatitis virus B and C [29,30], liver sinusoidal endothelial cells [31], brain endothelial cells [32], peritoneal macrophages [33] and cancer cells [34,35]. For tumor endothelial cell (TEC)-targeting, we modified the YSK-MEND with a cyclic RGD peptide (RGD-MEND), which is known to be a ligand against the $\alpha_V\beta_3$ integrin heterodimer, which is highly expressed in TECs and some types of cancer cells [36]. We previously reported that the RGD-MEND had the ability to suppress mRNA and protein expression specifically in TECs (ED50: 0.75 mg siRNA/kg) [37,38].

In this manuscript, we report on an investigation of the effect of VEGFR2 inhibition on altering the intratumoral distribution of LPs and a tumor microenvironment with a clustered-vessel type cancer model by the RGD-MEND. We infer from these results that clustering cell types with regard to a relative position between tumor vessels and stroma might pose a difference in the response to altering tumor microenvironment therapy, including anti-angiogenic therapy.

2. Materials and methods

2.1. Materials

Phosphate buffered saline without Ca^{2+} or Mg^{2+} (PBS (−)) was purchased from Wako Pure Chemical Industries (Osaka, Japan). Cholesteryl 3-*N*-(dimethylaminoethyl)carbamate hydrochloride (DC-chol) was purchased from AVANTI Polar Lipids (Alabaster, AL, USA) Distearoyl-*sn*-glycerophosphocholine (DSPC), polyethyleneglycol2000 (PEG) – distearoyl-*sn*-glycerophosphoethanolamine (PEG-DSPE), PEG-dimyristoylglycerol (PEG-DMG) and PEG-distearoylglycerol (PEG-DSG) were obtained from the NOF CORPORATION (Tokyo Japan). Cholesterol (chol), cholesterol hemisuccinate (CHEMS), TRI Reagent, Hoechst33342, RPMI-1640 and DMEM were purchased from Sigma-Aldrich (St. Louis, MO, USA). 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate (DiI) and 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindodicarbocyanine (DiD) were obtained from PromoKine (Heidelberg, Germany). [^{3}H]-cholesteryl hexadecyl ether (CHE), Soluene-350 and Hionic-Fluor were purchased from PerkinElmer (Waltham, MA, USA). FITC-labeled isolectin B4 (GSIB4) was obtained from Vector Laboratories (Burlingame, CA, USA) the High-Capacity RNA-to-cDNA kit and Alexa647-labled GSIB4 were purchased from Thermo Fisher Scientific (Waltham, MA, USA). YSK05 was synthesized as previously described [24]. Primers were purchased from Sigma-Aldrich Japan (Ishikari, Japan), and all of the primer sets are listed in Supplemental Table 1. Anti-mouse CD16/32 IgG (101302), PE anti-human HLA-A,B,C antibody (311406) and anti-mouse VEGFR2 rat IgG (136402) were obtained from BioLegend (San Diego, CA, USA). Anti-mouse collagen Type 1, α1 (COL1A1) rabbit polyclonal antibody was purchased from NOVUS Biologicals (Minneapolis, MN, USA). All of siRNAs were synthesized at Hokkaido System Science (Sapporo, Japan), and were listed in Supplemental Table 2. Cyclic RGD peptide was purchased from Peptides international (Louisville, KY, USA). OS-RC-2 (human renal cell carcinoma, RCC), Huh-7 (human hepatocellular carcinoma, HCC) and HCT116 (human colorectal cancer, CRC) were purchased from the ATCC.