Journal of Controlled Release 261 (2017) 275–286

Contents lists available at ScienceDirect

Journal of Controlled Release

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

cRGD peptide installation on cisplatin-loaded nanomedicines enhances efficacy against locally advanced head and neck squamous cell carcinoma bearing cancer stem-like cells

Kazuki Miyano[a], Horacio Cabral[b], Yutaka Miura[c], Yu Matsumoto[a], Yuki Mochida[d], Hiroaki Kinoh[d], Caname Iwata[e], Osamu Nagano[f], Hideyuki Saya[f], Nobuhiro Nishiyama[d,g], Kazunori Kataoka[b,c,d,h,*], Tatsuya Yamasoba[a]

[a] Department of Otolaryngology and Head and Neck Surgery, Graduate School of Medicine and Faculty of Medicine, The University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo 113-8655, Japan
[b] Department of Bioengineering, Graduate School of Engineering, The University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo 113-8656, Japan
[c] Division of Clinical Biotechnology, Center for Disease Biology and Integrative Medicine, Graduate School of Medicine, The University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo 113-0033, Japan
[d] Innovation Center of Nanomedicine, Kawasaki Institute of Industry Promotion, 66-20 Horikawa-cho, Saiwai-ku, Kawasaki 212-0013, Japan
[e] Department of Molecular Pathology, Graduate School of Medicine and Faculty of Medicine, The University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo 113-0033, Japan
[f] Division of Gene Regulation, Institute for Advanced Medical Research, School of Medicine, Keio University, 35 Shinanomachi, Shinjuku-ku, Tokyo 160-8582, Japan
[g] Laboratory for Chemistry and Life Science, Institute of Innovative Research, Tokyo Institute of Technology, R1-11, 4259 Nagatsuta, Midori-ku, Yokohama 226-8503, Japan
[h] Policy Alternatives Research Institute, The University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo 113-1709, Japan

ARTICLE INFO

Keywords:
Drug delivery
Polymeric micelles
Head and neck carcinoma
cRGD peptide
Integrin
CD44-variant
Tongue cancer

ABSTRACT

Recalcitrant head and neck squamous cell carcinoma (HNSCC) usually relapses after therapy due to the enrichment of drug resistant cancer stem-like cells (CSCs). Nanomedicines have shown potential for eradicating both cancer cells and CSCs by effective intratumoral navigation for reaching particular cell populations and controlling drug delivery. The installation of ligands on nanomedicines is an attractive approach for improving the delivery to CSCs within tumors, though the development of CSC-selective ligand-receptor systems has been challenging. Herein, we found that the CSC subpopulation in HNSCC cells overexpresses $\alpha_v\beta_5$ integrins, which is preferentially expressed in tumor neovasculature and cancer cells, and can be effectively targeted by using cyclic Arg-Gly-Asp (cRGD) peptide. Thus, in this study, we propose installing cRGD peptide on micellar nanomedicines incorporating cisplatin for improving their activity against CSCs and enhancing survival. Both cisplatin-loaded micelles (CDDP/m) and cRGD-installed CDDP/m (cRGD-CDDP/m) were effective against HNSCC SAS-L1-Luc cells *in vitro*, though cRGD-installed CDDP/m was more potent than CDDP/m against the CSC fraction. *In vivo*, the cRGD-CDDP/m also showed significant antitumor activity against HNSCC orthotopic tumors, *i.e.* SAS-L1 and HSC-2. Moreover, cRGD-CDDP/m rapidly accumulated into the lymph node metastasis of SAS-L1 tumors, effectively inhibiting their growth, and prolonging mice survival. These findings indicate cRGD-installed nanomedicines as an advantageous strategy for targeting CSCs in HNSCC, and particularly, cRGD-CDDP/m as a significant therapeutic strategy against regionally advanced HNSCC.

1. Introduction

Head and neck squamous cell carcinoma (HNSCC) is a severe cancer accounting for > 800,000 new cases and 360,000 deaths worldwide every year [1]. As HNSCC arises in organs related to breathing, swallowing and speaking, radical surgeries would inevitably compromise vocal and/or deglutition functions. Recent recommended therapies involve induction chemotherapy and concurrent chemoradiotherapy to preserve these functions, but serious toxicities can be encountered in many patients [2–6]. Besides, after an initial response to these therapies, most HNSCC eventually relapse. This recurrence has been associated with resistant cancer stem-like cells (CSCs) sub-populations in the tumors, which are not susceptible to traditional therapies [7–9]. Therefore, safe and effective therapeutics strategies against HNSCC that

* Corresponding author at: Innovation Center of Nanomedicine, Kawasaki Institute of Industry Promotion, 66-20 Horikawa-cho, Saiwai-ku, Kawasaki 212-0013, Japan.
E-mail address: kataoka@bmw.t.u-tokyo.ac.jp (K. Kataoka).

http://dx.doi.org/10.1016/j.jconrel.2017.06.021
Received 2 February 2017; Received in revised form 13 June 2017; Accepted 22 June 2017
Available online 27 June 2017

are capable of tackling both cancer cells and CSCs are essential for improving patients' survival.

Nanomedicines have shown high potential for developing effective antitumor therapies by efficiently delivering the anticancer drugs to their targets. Among promising nanomedicines, drug-loaded polymeric micelles, *i.e.* core-shell nanoassemblies of block copolymers encapsulating bioactive molecules, have demonstrated outstanding features for tumor targeting [10,11], and several polymeric micelles incorporating anticancer drugs are being evaluated in human clinical studies, displaying increased efficacy and reduced side effects [12]. Particularly, cisplatin-loaded micelles (CDDP/m) [13], which are under phase III clinical trials against pancreatic cancer under the development name NC-6004 (Nanocarrier Co., Ltd), have shown significant activity against HNSCC models [14] by overcoming the glutathione-mediated drug resistance in the CSCs fraction, that is, the sub-population expressing a variant isoform of CD44 (CD44v) [15–17], through enhanced CDDP delivery to nuclear DNA after endocytosis of the micelles. Considering that the intracellular delivery of nanomedicines to particular cell populations can be increased by installing ligand molecules on the surface of nanomedicines [18,19], the modification of CDDP/m with ligands directed to particular markers expressed by the CSC subpopulation of HNSCC cells could further improve therapeutic outcomes.

The development of ligand-installed nanomedicines for delivering cytotoxic agents to CSCs has been challenging, as the potential receptors could also be found in normal cells, such as adult progenitor cells, and the expression of the receptors on CSCs may vary between patients [20]. Notably, histological studies of human HNSCC have shown that $\alpha_v\beta_5$ integrins, which have been linked with cancer stemness, metastasis and drug resistance in several malignancies [21], are highly expressed on HNSCC cells [22,23]. In this study, we confirmed the expression of $\alpha_v\beta_5$ integrins on the CD44v-positive fractions of two human oral carcinoma cells, *i.e.* HSC-2 and SAS-L1. Therefore, by installing the $\alpha_v\beta_5$ integrin-targeting cyclic Arg-Gly-Asp (cRGD) peptide on the surface of CDDP/m, we aimed to develop therapeutic strategies with improved efficacy against HNSCC bearing CSCs. Our results showed that cRGD-installed CDDP/m (cRGD-CDDP/m) have significant antitumor activity against both orthotopic HSC-2 and SAS-L1-Luc tumors, and rapidly and selectively accumulated in the lymph node metastases of the SAS-L1-Luc tumor and reduced their growth rate. Thus, cRGD-CDDP/m is a noteworthy translationable approach for the treatment of locally advanced HNSCC.

2. Materials and methods

2.1. Materials

cis-Diamineplatinum(II) dichloride (Sigma-Aldrich Co., St Louis, MO, USA), AlexaFluor 555-succinimidyl ester, Alexa Fluor 647-succinimidyl ester (Life Technologies Corporation, Carlsbad, CA, USA), cyclo [RGDfK(CX-)] (cRGD peptide, X = 6-aminocaproic acid: ε-Acp, Peptide Institute Inc., Osaka, Japan), and other regents were used without further purification. Minimum Essential Medium (MEM) containing non-essential amino acids without glutamine was purchased from Life Technologies Corporation (Carlsbad, CA, USA). Dulbecco's Modified Eagle's Medium (DMEM)-high glucose was purchased from Sigma-Aldrich (St. Louis, MO, USA). Fetal bovine serum (FBS) was purchased from Sumitomo Dainippon Pharma Co. (Osaka, Japan). Phosphate buffered saline (PBS) was purchased from WakoPure Chemical Industries (Osaka, Japan). Cell Counting Kit-8 was purchased from Dojindo Laboratories (Kumamoto, Japan). CellTiter-Glo 3D cell Viability Assay and luciferin were purchased from Promega (Fitchburg, WI, USA). Accutase was purchased from Innovative Cell Technologies Inc. (San Diego, CA, USA). Anti-integrin $\alpha_v\beta_3$ antibody and anti-integrin $\alpha_v\beta_5$ antibody were purchased from R & D systems Inc. (Minneapolis, MN, USA). Anti-integrin β_3 antibody was purchased from Cell Signaling Technology (Danvers, MA, USA). Anti-mouse integrin $\alpha_5\beta_1$ antibody was purchased from Merck Millipore Co. (Darmstadt, Germany). Anti Human CD44v9 [Clone: RV3] was purchased from Cosmo Bio Co., Ltd. (Tokyo, Japan). Alexa Fluor 488-conjugated donkey anti-rat IgG H & L preabsorbed, Alexa Fluor 647-conjugated goat anti-mouse IgG H & L preabsorbed, Alexa Fluor 488-conjugated anti-rabbit IgG H & L, rat IgG2a, kappa monoclonal [RTK2758] - Isotype Control, and mouse IgG1, kappa monoclonal [15-6E10A7] - Isotype Control were purchased from abcam (Cambridge, UK). Biotinylated anti-rat IgG and VECTASTAIN Elite ABC Reagent were purchased from Vector laboratories Inc. (Burlingame, CA, USA). Peroxidase-conjugated anti-rabbit IgG was purchased from Nichirei Biosciences Inc. (Tokyo, Japan). TSA Cyanine 3 System was purchased from Perkin Elmer (Waltham, MA, USA). Anti-CD31 antibody was purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA). Isoflurane was purchased from Pfizer Inc. (New York, NY, USA). Matrigel Matrix was purchased from BD Biosciences (Flanklin Lakes, NJ, USA).

2.2. Cell lines and animals

SAS-L1 and SAS-L1-GFP cells, human tongue carcinoma cells with high lymph node metastatic potential, were provided by the Department of Oral and Maxillofacial Surgery, School of Dentistry, Showa University (Tokyo, Japan). SAS-L1 cells were sequentially infected with lentiviral vectors carrying EF promoter-driven luciferase to establish SAS-L1-Luc cells stably expressing luciferase. SAS-L1, SAS-L1-GFP, and SAS-L1-Luc cells were maintained in DMEM-high glucose plus 10% FBS, 100 U/ml penicillin G, and 100 μg/ml streptomycin. HSC-2 cells, human oral carcinoma, were purchased from RIKEN BRC (Tsukuba, Japan). HSC-2 cells were maintained in MEM media plus 10% FBS. BALB/c nu/nu female mice, 6-weeks, were purchased from Charles River Co. (Tokyo, Japan). All animal experiments were conducted under the ethical guidance of The University of Tokyo.

2.3. Preparation of polymeric micelles

Poly(ethylene glycol)-*b*-poly(L-glutamic acid) (MeO-PEG-*b*-P(Glu): molecular weight of PEG ($M_{n,PEG}$) = 12,000, polymerization degree of P(Glu) ($DP_{P(Glu)}$) = 40) and maleimide-conjugated poly(ethylene glycol)-*b*-poly(L-glutamic acid) (Mal-PEG-*b*-P(Glu): $M_{n,PEG}$ = 12,000, $DP_{P(Glu)}$ = 40) were synthesized as previously reported [18,19,24–26]. The ω-amino groups of MeO-PEG-*b*-P(Glu) was fluorescently labeled by conjugating the Alexa 555 or Alexa 647 succinimidylesters. cRGD-CDDP/m was prepared as follows: MeO-PEG-*b*-P(Glu) and Mal-PEG-*b*-P(Glu) copolymers (3:1 M ratio) were dissolved in distilled water containing CDDP ([Glu] = 5 mM, [CDDP]/[Glu] = 1.0, [MeO-PEG-*b*-P(Glu)]/[Mal-PEG-*b*-P(Glu)] = 1.0). These solutions were filtrated by polyvinylidene fluoride membrane (Millipore, pore size: 0.22 μm), and stirred at 37 °C for 120 h. The reaction solutions were transferred to a dialysis membrane (Spectra/Pro 6 membrane; Molecular weight cut-off (MWCO): 6000–8000) and dialyzed for 24 h against distilled water, followed by ultrafiltration (MWCO: 30,000) to obtain maleimide-CDDP/m (Mal-CDDP/m). For cRGD conjugation, cyclo[RGDfK(CX-)] (0.640 mM) was added to Mal-CDDP/m solutions and stirred for 20 h at 37 °C. This solution was purified by ultrafiltration (MWCO: 30,000) to obtain cRGD-CDDP/m. The amount of cRGD on the surface of micelles was fixed at 20% (a molar basis) of the total PEG chains based on our previous report [18]. CDDP/m was prepared from MeO-PEG-*b*-P(Glu) by following the same manner as described above. The diameter of CDDP/m and cRGD-CDDP/m was determined to be 30 nm by DLS measurements at 25 °C by using a Zetasizer Nano ZS90 (Malvern Instruments, Malvern, UK). The platinum content of the CDDP/m and cRGD-CDDP/m was determined by inductively coupled plasma mass spectrometry (ICP-MS) using 7700 × ICP-MS systems (Agilent Technologies, Santa Clara, CA, USA). To prepare Alexa 555-labeled cRGD-CDDP/m and Alexa 647-labeled CDDP/m, MeO-PEG-*b*-P(Glu)-Alexa 555 or MeO-PEG-*b*-P(Glu)-Alexa 647 was used instead of MeO-PEG-*b*-P