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A free cysteine prolongs the half-life of a homing peptide and improves its tumor-penetrating activity

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ABSTRACT

The accessibility of extravascular tumor tissue to drugs is critical for therapeutic efficacy. We previously described a tumor-targeting peptide (iRGD) that elicits active transport of drugs and macromolecules (covalently coupled or co-administered) across the vascular wall into tumor tissue. Short peptides (iRGD is a 9-amino acid cyclic peptide) generally have a plasma half-life measured in minutes. Since short half-life limits the window of activity obtained with a bolus injection of iRGD, we explored to extend the half-life of the peptide. We show here that addition of a cysteine residue prolongs the plasma half-life of iRGD and increases the accumulation of the peptide in tumors. This modification prolongs the activity of iRGD in inducing macromolecular extravasation and leads to greater drug accumulation in tumors than is obtained with the unmodified peptide. This effect is mediated by covalent binding of iRGD to plasma albumin through a disulfide bond. Our study provides a simple strategy to improve peptide pharmacokinetics and activity. Applied to RGD, it provides a means to increase the entry of therapeutic agents into tumors.

1. Introduction

The efficacy of current cancer therapies is limited by poor penetration of drugs into tumor tissue and adverse effects on healthy tissues. Despite their increased permeability, tumor vessels of many solid tumors form a barrier for passively diffusing therapeutics, especially proteins or nanoparticles [1]. We previously identified a novel tumor targeting peptide, iRGD (CRGDK/RGPD/EC), which is a cyclic peptide linked by a disulfide bond [1]. Like conventional RGD-containing peptides, iRGD initially binds to αv integrins upregulated on the endothelial wall of many tumors. Sequential proteolytical cleavage generates CRGDK/R, which dissociates from integrins but gains affinity for another tumor vessel signature molecule, neuropilin-1 (NRP1) [1,2]. NRP1 binding triggers an active transport across the blood vessels *in vivo*, enabling the penetration of iRGD and its coupled cargo deep into the extravascular tumor parenchyma. Importantly, this process allows the tumor penetration of cargo that is nearby but not chemically coupled to iRGD (bystander activity) [3]. Thus iRGD can be used in combination with approved drugs to improve their therapeutic index. Co-administration with iRGD has been shown to increase tumor extravasation of multiple types of therapeutics, including small molecule drugs (*e.g.* doxorubicin), an antibody drug (trastuzumab) and therapeutic nanoparticles (nab-paclitaxel) [3].

A major limitation to the therapeutic application of peptides is their short half-life in circulation, which is primarily caused by renal clearance [4], and this also applies to iRGD. A common strategy in prolonging the blood half-life of peptides, and other small molecules, is to increase the molecular weight above the renal clearance limit by coupling the peptide to a carrier molecule such as serum albumin. A way of accomplishing albumin coupling is to conjugate the peptide to the sole reactive cysteine sulfhydryl group in the albumin molecule through a disulfide bond [5,6]. This reaction can take place *in vivo* upon intravenous injection of a compound that contains a free sulfhydryl group [7], which in a peptide can be provided by adding a cysteine residue. An attractive feature of this procedure is that the therapeutic compound is still a simple peptide rather than a high molecular weight conjugate. This approach has not been tested with homing peptides. Here, we applied this strategy to iRGD to determine whether incorporation of an extra cysteine residue provides benefits regarding the pharmacokinetics and tumor-penetrating activity of iRGD.

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2. Materials and methods

2.1. Animal models and reagents

Wild type (WT) Balb/c mice were used in all experiments. To generate tumors, 4T1 mouse breast cancer cells (~1 × 10^6 cells/mouse) were injected into the mammary fat pad of female mice, and the animals were used for experiments when tumors reached ~1 cm in diameter. Cyclic peptides containing an extra cysteine in addition to the two cysteines forming the cyclizing bond were synthesized in our laboratory [1,3]. C-GGS-H6 with fluorescein (FAM) conjugated at the N-terminus (Fig. 4A), and iRGD with FAM at the N-terminus and a fluorescence quencher, dabcyl (4-((4-(dimethylamino)phenyl)azo)benzoic acid) at the C-terminus (q-iRGD) (Fig. 1A), were custom-synthesized by a commercial manufacturer (Lifetein). The other peptides were also labeled with FAM, which was placed at the N-terminus with 6-aminohexanoic acid as a spacer separating the dye and the extra cysteine from the peptide sequence.

2.2. In vitro peptide stability

Blood was drawn from WT Balb/c mice, diluted 3 fold with PBS (pH = 7.4), centrifuged at 500 ×*g* for 5 min to remove the cells and obtain diluted plasma. q-iRGD (1.5 μmol) was incubated in 60 μl of the plasma for the indicated times at 37 °C or 4 °C. As a positive control for enzymatic degradation, trypsin (25 mg/L) was added and incubated for 1 h at 37 °C. Dithiothreitol (DTT; 100 mM) was added to the samples to reduce the q-iRGD disulfide bond. The fluorescent signal was quantified using FlexStation fluorescent plate reader (Molecular Devices). After subtracting the background signal (uncleaved peptide), the fluorescence intensity was normalized to that of the trypsin treated sample to obtain the fraction of cleaved peptide.

2.3. Plasma half-life measurement

FAM-labeled peptides (200 μg in 100 μl PBS) were intravenously injected into the tail vein of normal Balb/c mice. Five microliters of blood was drawn from tail vein at the indicated time points and immediately diluted 100 fold in PBS containing 5 mM EDTA. The diluted blood was then centrifuged at 500 ×*g* for 5 min and the fluorescent signal in the supernatant was quantified with FlexStation fluorescent plate reader (Molecular Devices). The area under the curve was calculated as previously described [8].

The fluorescent intensity of FAM-labeled peptides at the indicated time points was normalized to the 2-min value as the fraction of the peptide remaining in the circulation. The kinetic data for all peptides were fitted against one-phase decay model using Prizm software (GraphPad Software, Inc.).

2.4. Tumor homing

FAM-labeled peptides (200 μg in 100 μl PBS) were intravenously injected into tail vein of mice and allowed to circulate for 1 h before the animals were anesthetized and perfused with PBS containing 1% (w/v) BSA as described [1]. Major organs were excised and the fluorescent intensity of the whole organs was analyzed using the Illumatool Bright Light System LT-9900 (Light tools, Inc.). The tissues were then processed for fluorescence microscopy.

2.5. Evans Blue assay

Peptides (200 μg in 100 μl PBS) were intravenously injected into mice bearing 4T1 tumors and allowed to circulate for different periods of time. Evans Blue (EB) dye (1 mg in 100 μl PBS) was then injected and allowed to circulate for another 40 min. The mice were anesthetized and perfused through the heart with PBS containing 1% BSA, and major organs were excised and weighed. The dye was extracted from tissues in N,N-dimethylformamide for 24 h at 37 °C and quantified by measuring absorbance at 600 nm in a spectrophotometer.

2.6. Quantification of drug accumulation in tumors

Mice bearing 4T1 tumors were first injected with iRGD or Cys-X-iRGD (200 μg in 100 μl PBS), and 15 min later with trastuzumab (1.5 mg/kg) or nab-paclitaxel (1.5 mg/kg). Intracardiac perfusion was performed 24 h later, major organs were collected and processed for immunofluorescence analysis. The drug content was quantified from the fluorescent signal and normalized by the cell number within the images (determined by ImageJ) to obtain relative drug content per cell.

2.7. Immunofluorescence analysis

Tissues were fixed with 4% paraformaldehyde (PFA) in PBS (PH = 7.4) for 2 h and dehydrated with 20% sucrose in PBS for 48 h. The tissue samples were embedded in O.C.T (Tissue-Tek) and frozen on dry ice followed by sectioning [3].

Tumor homing and extravasation of peptides were analyzed using the following primary antibodies: rabbit anti-FITC antibody (A889, Molecular Probes), rabbit anti-His6 tag antibody (sc-803, Santa Cruz Biotechnology), rat anti-mouse CD31 primary antibody (553370, BD Pharmingen). The secondary antibodies were: donkey anti-rabbit secondary antibody (A21206, Molecular Probes) and donkey anti-rat secondary antibody (712-165-153, Jackson ImmunoResearch). Rabbit anti-human IgG

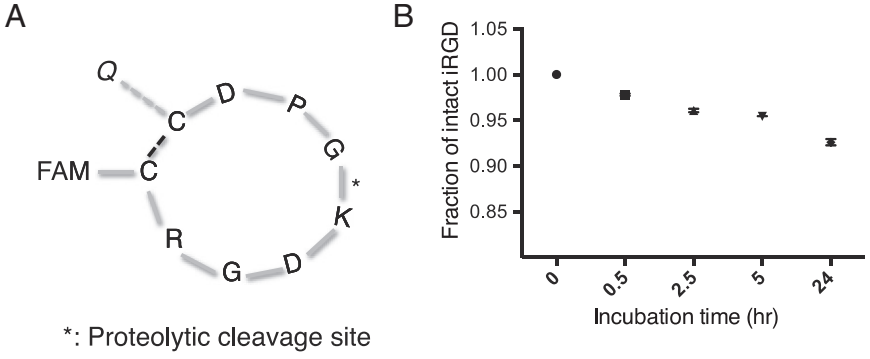

Fig. 1. *In vitro* stability of iRGD in mouse plasma. (A) The structure of q-iRGD. The disulfide bond is shown in dashed line. Q represents the dabcyl group (fluorescent quencher). The proteolytic cleavage site to activate iRGD tissue penetration is indicated by an asterisk. (B) Peptide stability measurement. Mouse plasma was obtained from normal mice (n = 3). A fixed amount of q-iRGD was incubated with mouse plasma for the indicated times at 37 °C, or at 4 °C for the 0 time point. The fraction of intact q-iRGD was obtained as described in the Materials and methods section. Plasma from the three mice was tested separately. The values are mean ± SEM (standard error of mean).

(H + L) antibody (31786; Thermo) and rabbit anti-human albumin antibody (ab2406; Abcam) were used to detect trastuzumab and albumin, respectively.

2.8. Immunoprecipitation

To isolate proteins that bind injected peptides in the blood, peptide (200 μg in 100 μl PBS) was intravenously injected into the tail vein of mice bearing 4T1 tumors. Blood was drawn 5 min later, and the plasma was isolated as described above and incubated with antibodies to capture the injected peptides and any coupled proteins. Cys-iRGD and iRGD were captured with rabbit anti-FITC antibody A889, and protein A/G magnetic beads (Pierce) were then used to isolate the immune complexes, followed by proteomics analysis at the proteomics core facility of Sanford-Burnham Medical Research Institute.

In a separate experiment, mice bearing 4T1 tumors were injected with C-GGS-H6 and plasma was processed as described above, except that goat anti-mouse albumin antibody (PA1-30899, Thermo) was used to precipitate albumin and C-GGS-H6 peptide that had become conjugated to it. The supernatant was further incubated with Ni beads (Pierce) to isolate any C-GGS-H6-conjugated proteins other than albumin. The isolated proteins were separated on SDS-PAGE and immunoblotted using rabbit anti-FITC antibody A889 and IRDye donkey anti-rabbit secondary antibody (Rockland).

2.9. In vitro albumin and dextran conjugation

Mouse albumin (55858, MP Biomedicals) or amine dextran (D1861, Invitrogen) was incubated in PBS with Bis-maleimide-PEG_3 linker (22337, Pierce) or NHS-PEG 2000-maleimide linker (A5001-1, JenKem), respectively, for 1 h at RT (in a 10-fold molar excess of the linker). Excess linker was removed using a dialysis cassette (MWCO = 3500 for Bis-maleimide-PEG3; MWCO = 30,000 for NHS-PEG 2000-maleimide) at 4 °C for 4 h. C-GGS-H6 peptide was then conjugated to albumin and dextran by incubating a 10-fold excess of the peptide with the linker-reacted compounds for 1 h at RT. Unconjugated peptide was removed by dialysis. Free FAM-labeled peptide was dialyzed in parallel to confirm the clearance of unconjugated peptide.

3. Results

We first investigated whether iRGD is sensitive to enzymatic degradation in the plasma by testing q-RGD, an iRGD with FAM at one end and a fluorescence quencher at the other (Fig. 1A). Proteolytic cleavage of iRGD together with the reduction of the disulfide bond is expected to separate FAM from the dabcyl quencher group and thus rescue the fluorescent signal. q-iRGD remained >90% stable in mouse plasma for at least 24 h (Fig. 1B). This is much longer than the plasma half-life of iRGD (8 min, Table S1), indicating that proteolytic cleavage of iRGD is not a significant factor in determining the half-life of the peptide.

We next sought to extend the plasma half-life of iRGD. We added a cysteine residue to the peptide (Cys-iRGD), the free sulfhydryl group of which provided a potential coupling group for conjugation to plasma albumin, and perhaps to some other blood components as well (Fig. 2A). Cys-iRGD exhibited a longer plasma half-life (22 min) than parental iRGD (Fig. 2B and C; Table S1). Tumor accumulation of Cys-iRGD was also more robust than that of iRGD, especially when measured 3 h after the injection, at which time iRGD signal in the tumors had weakened (Fig. S1). After intravenous injection, the FAM signal from both peptides was exclusively present in plasma, indicating no significant association with blood cells (Fig. S2).

Next, we investigated whether the bystander activity of iRGD [3] would also be augmented by the extra cysteine. We used the Evans Blue (EB) dye to monitor plasma albumin extravasation into 4 T1 tumors as an indicator of the bystander activity [3,9]. As the bystander activity is our focus here, we used perfusion through the heart to eliminate blood content and to focus the analysis to the entry of the peptides and their cargo into the extravascular space. iRGD significantly increased EB extravasation into tumor tissue over the PBS control at 30 min after the injection, but Cys-iRGD was inactive (Fig. 3A). To

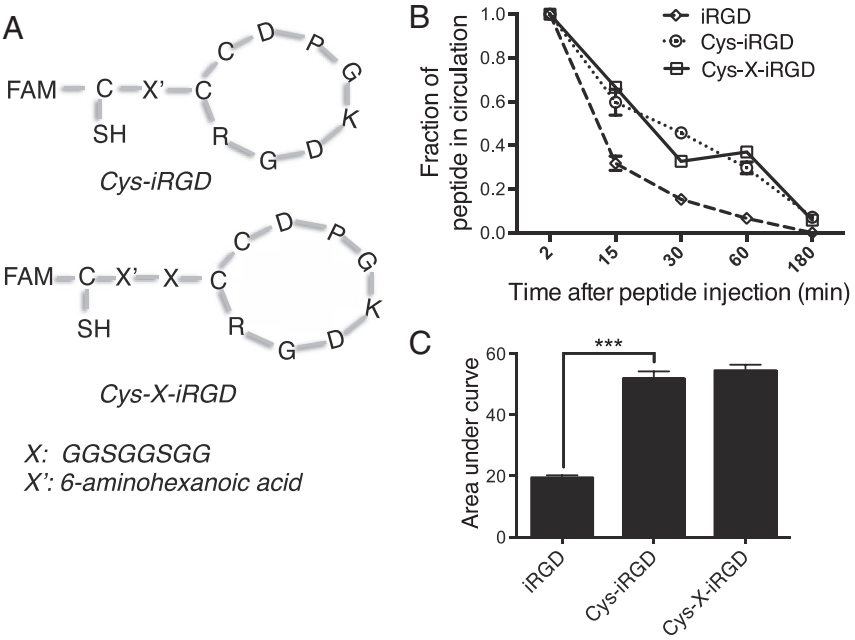

Fig. 2. Extra cysteine extends the plasma half-life of iRGD. (A) The structure of the iRGD variants used in this study. (B) Plasma half-life measurement. All peptides were labeled with FAM at the N-terminus. Each peptide was injected into normal mice (n = 3), and the fluorescence intensity of each peptide in plasma at the indicated time points was normalized to that at 2 min as the y-axis values. Each peptide was tested separately in three mice and the mean values (±SEM) are shown. (C) Area under the curve (AUC). The AUC values of the curves in (B) were calculated as described in the Materials and methods section. ***, p value < 0.001 (Student t-test).

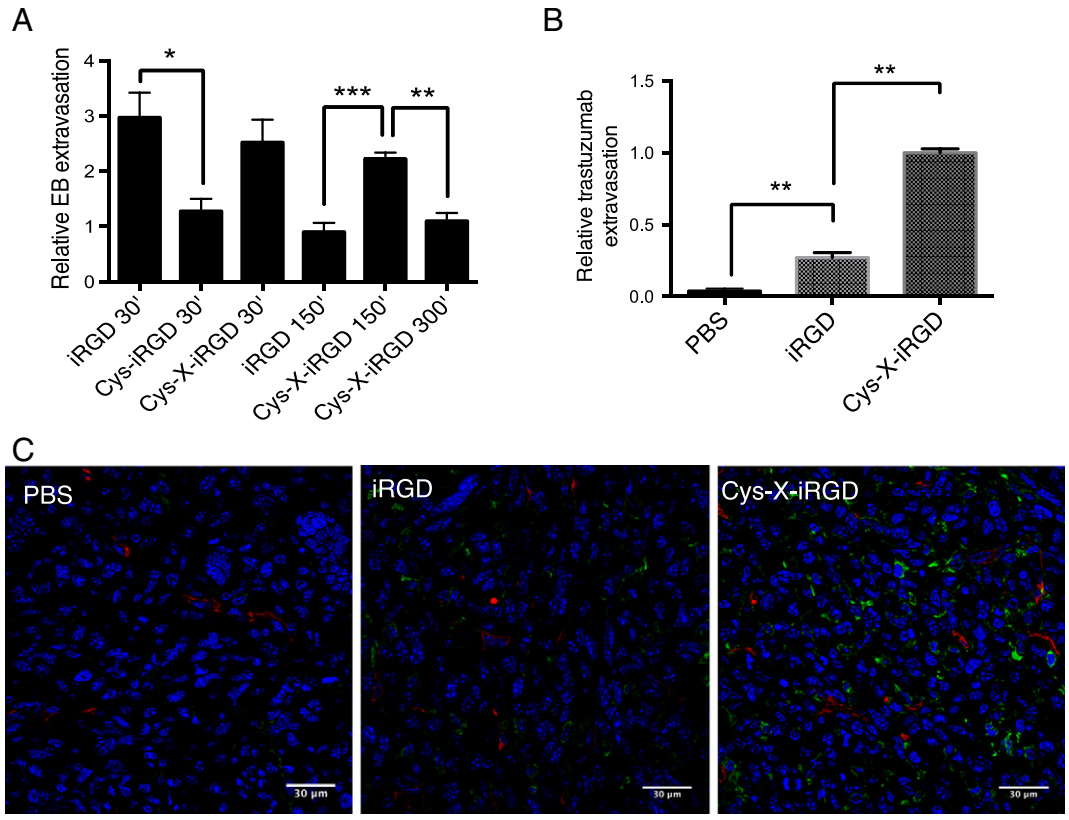

Fig. 3. Addition of a cysteine prolongs the bystander activity of iRGD and increases drug extravasation into tumors. Extravasation of Evans Blue (A) and trastuzumab (B) was measured after prior injection of iRGD variants. (A) Each peptide was injected into mice bearing 4T1 tumors (n = 3–5) for the EB assay. The time interval between the injection of the peptide and EB administration was varied as shown on the x-axis. The amount of EB recovered from tumor tissue, measured as OD_{600} per mg of tissue, was determined and is shown normalized to a PBS injection control. Error bars indicate SEM. (B) The indicated peptides were injected into mice bearing 4T1 tumors (n = 3) together with trastuzumab, and major organs including tumors were collected after 24 h of circulation. Trastuzumab extravasation into tumor tissues was detected by anti-human IgG antibody, and the fluorescent intensity per cell was quantified and normalized to that of PBS control as relative trastuzumab extravasation. Error bars indicate SEM. Asterisks (*, **, ***) indicate $p < 0.05$, 0.01 and 0.001, respectively (Student t test). (C) Representative images of trastuzumab accumulation in tumors. Trastuzumab was detected using anti-human IgG antibody (green), and blood vessels and nuclei were stained using rat anti-mouse CD31 (red) and DAPI (blue).

rescue the bystander activity of Cys-iRGD, we increased the linker length between the extra cysteine and iRGD by adding a GGSGGSGG linker (Cys-X-iRGD) (Fig. 2A). Cys-X-iRGD exhibited a similar kinetic profile in blood as Cys-iRGD (Fig. 2 B and C, Table S1), but had regained the bystander activity at 30 min (Fig. 3A). Moreover, Cys-X-iRGD remained active for a longer time (150 min) after injection, at which time iRGD had lost almost all of its activity (Fig. 3A). The bystander activity of Cys-X-iRGD was undetectable 5 h after the injection (Fig. 3A).

To determine whether the prolonged bystander activity might provide a therapeutic benefit, we compared the effects of Cys-X-iRGD and iRGD on 24-h drug extravasation into tumors. Both peptides induced more trastuzumab accumulation outside tumor blood vessels than the control (PBS), but Cys-X-iRGD was significantly more potent in this regard than iRGD (Fig. 3B and C). Trastuzumab concentration in other organs was not elevated by either peptide (Fig. S3). Similar results were obtained when nab-paclitaxel was used as the drug (Fig. S4).

To gain more information on the role of individual plasma proteins in the capture and transport of peptides with a free thiol group, we set out to identify specific plasma carriers for Cys-iRGD. After circulation in the blood for 5 min, FAM-labeled peptides were precipitated with anti-FITC antibody, and the covalently bound proteins were identified by proteomics analysis (File S1). Albumin was the most abundant protein bound to Cys-iRGD and it exhibited the biggest difference between Cys-iRGD and the iRGD control. To confirm this observation and rule out any contribution by iRGD in binding plasma components, we synthesized a FAM-labeled peptide that contains a cysteine residue and a hexa-histidine (H6) tag (Fig. 4A). The cysteine and the tag were connected through the same linker we used for Cys-X-iRGD, except that a lysine residue was added to allow fixation of the peptide in tissue sections. This peptide (C-GGS-H6) had a similar kinetic profile in the blood as Cys-iRGD (Fig. 4B, Table S1).

Immunoprecipitation of plasma drawn after an intravenous injection of C-GGS-H6 with an anti-mouse albumin antibody followed by immunoblotting with anti-FITC revealed a fluorescent albumin band, indicating that C-GGS-H6 had become covalently conjugated to albumin (Fig. 4C). Conjugation of C-GGS-H6 to albumin was seen already 5 min after the injection. The complex was sensitive to treatment with a reducing agent, indicating that a disulfide bond had formed between the peptide and albumin (Fig. 4C). We then made use of the his-tag to isolate all other plasma proteins conjugated to C-GGS-H6 from the supernatant after albumin depletion. Only a trace amount of fluorescence was detected in this fraction (Fig. 4C), suggesting that the vast majority of C-GGS-H6 was conjugated to albumin.

As an alternative assay, we irreversibly coupled C-GGS-H6 to albumin *in vitro* using a maleimide linker. Both the C-GGS-H6 peptide and the albumin conjugate (Alb-GGS-H6) exhibited similar tissue distribution, and also substantial extravasation into tumors similar to iRGD (Fig. 5A and B, S5). In contrast, C-GGS-H6 conjugated to dextran of a similar size as albumin exhibited diminished tumor extravasation (Fig. S6) and was largely confined within tumor vessels (Fig. 5B). These results show that an extra cysteine residue, through spontaneous conjugation to albumin, improves the pharmacokinetic properties of iRGD and enhances the iRGD-induced tumor extravasation of plasma molecules and drugs.

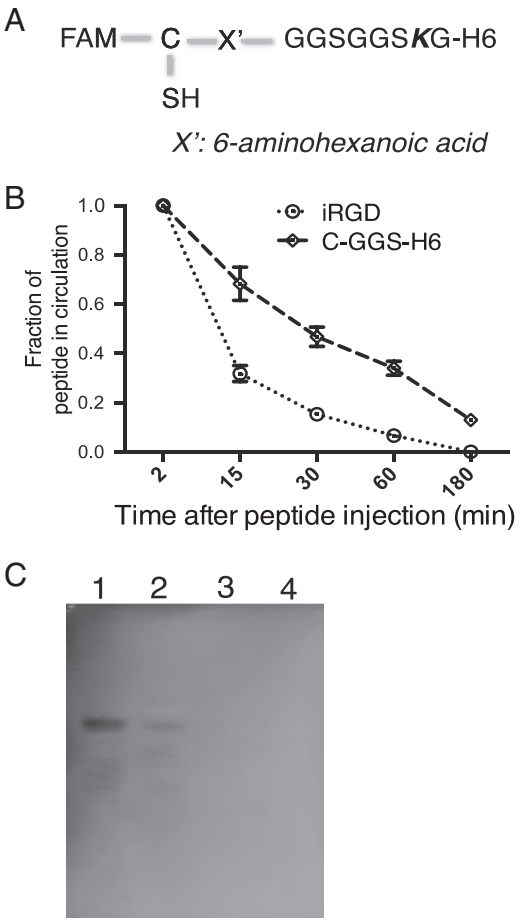

Fig. 4. Cysteine control peptide exhibits prolonged plasma half-life and predominantly becomes conjugated to albumin. (A) The structure of C-GGS-H6. (B) Plasma half-life of C-GGS-H6 in comparison with iRGD. FAM labeled peptides were injected into normal mice ($n = 3$) and the plasma half-lives were quantified as described in Fig. 2B. (C) Immunoprecipitation with C-GGS-H6 immunoblotting was performed as described in the Materials and methods section. Lanes: 1, albumin immunoprecipitate; 2, albumin immunoprecipitate after DDT treatment; 3, His-tag pull-down; 4, His-tag pull-down after DDT treatment.

Some of the improvement may be related to an ability of albumin to increase tumor homing of any albumin-bound molecule.

4. Discussion and conclusions

In this study, we showed that adding a cysteine residue with a free thiol group to a tumor-homing peptide (iRGD) prolongs its blood half-life and makes it more effective in tumor homing and penetration. Importantly, this modification increases the ability of iRGD to promote the penetration into extravascular tumor tissue of compounds that are not coupled to the peptide (bystander effect). We also show that incorporation of an extra cysteine to an inert peptide leads to the conjugation to plasma albumin upon systemic administration and renders the peptides capable of some degree of tumor homing and extravasation.

The conjugation of Cys-iRGD to albumin in the blood was to be expected in light of previous literature. Multiple plasma proteins contain cysteine residues with a free sulfhydryl group [10,11], the most abundant of which is the Cys-34 of albumin [5,12]. The vast majority, if not all, of naturally circulating homocysteines [12] as well as drugs carrying thiol groups [13,14] have been shown to couple to Cys-34 of albumin. We also found albumin to be the predominant protein that captures peptides with extra Cys in the circulation. The sensitivity of the complexes to treatment with a reducing agent indicated a formation of a disulfide bond between the extra Cys thiol and Cys-34. The formation of such complexes can explain several observations in this study. First, structural studies have shown that Cys-34 is buried in a pocket, which is 10–12 Å deep [6], potentially explaining the linker length dependence of the Cys-iRGD bystander activity; a longer linker projects iRGD farther from albumin, allowing the peptide to interact with its receptors (αv integrin) in tumors. This linker requirement in maintaining the ability of albumin-coupled compound to engage in other interactions we observed here has not been previously noted. Second, albumin conjugation is likely to be primarily responsible for the prolonged half-life and enhanced tumor homing of Cys-iRGD compared to iRGD. Third, the plasma half-life of Cys-iRGD remains shorter than that of mouse albumin (22 min versus 1.2 day [15]). This difference could be, at least partially, explained by instability of the disulfide-bond linking the peptide to albumin. Finally, while it is clear that peptides with the free cysteine predominantly became conjugated with albumin in the blood, our data do not exclude the possibility that less abundant plasma components might also form disulfide-bonds with the peptide. However, such complexes would be unlikely to be responsible for the tumor homing of the inert peptide we used (C-GGS-H6), because albumin was active in this regard, and the tumor homing properties of a minor complex would have to be very strong to make a difference.

iRGD is a prototypic member of a recently discovered class of tumor penetrating peptides [16]. These peptides can induce an active transport into extravascular tumor tissue of macromolecules that are not covalently coupled to the peptide (bystander activity [3]). An important result of this present study is that adding an extra cysteine to iRGD increases its bystander activity. Moreover, this effect, which we have shown to last for less than 1 h after a bolus injection in mice, was prolonged to 3 h when Cys-X-iRGD was used. One hour roughly equals six half-lives of iRGD, which may explain the short duration of the iRGD bystander effect. Alternatively, receptor down-regulation could also limit the duration of the effect of a single injection. The fact that we found Cys-X-iRGD to give a longer bystander response than the original iRGD suggest that half-life in the circulation is the main limiting factor of time window of iRGD activity. Indeed, Cys-X-iRGD induced more accumulation than iRGD of two long-circulating drugs (trastuzumab and nab-paclitaxel) in extravascular tumor regions, which should translate into a better therapeutic outcome. Other tumor penetrating peptides (LyP-1 [16], iNGR [17], etc.) may benefit from disulfide-mediated albumin hitchhiking similar to what we described here.

Albumin has attracted much attention as a drug carrier not only because of its lack of toxicity and immunogenicity, but also its preferential accumulation in tumors and inflamed tissues [5]. Albumin is captured by the cell surface receptor gp60, and by the secreted, matricellular protein SPARC, the expression of which is upregulated in many types of tumors [5,18,19]. Gp60 binding initiates a caveolae-mediated transcytosis process that allows albumin extravasation beyond tumor vessels [20]. This extravasating ability of albumin may provide an additional advantage over other methods when applied to tumor targeting.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.jconrel.2013.12.006.

Author contribution

H.B.P, G.B.B and E.R designed the experiments. V.R synthesized the peptides. H.B.P performed all experiments. G.B.B, K.N.S, T.T and Z.G.S provided technical support. H.B.P and E.R wrote the paper.

Conflict of interest disclosure

VRK, KNS, TT and ER have ownership interest (including patents) in CendR Therapeutics Inc. ER is also founder, chairman of the board, consultant/advisory board member of CendR Therapeutics Inc. No potential conflicts of interest were disclosed by the other authors.

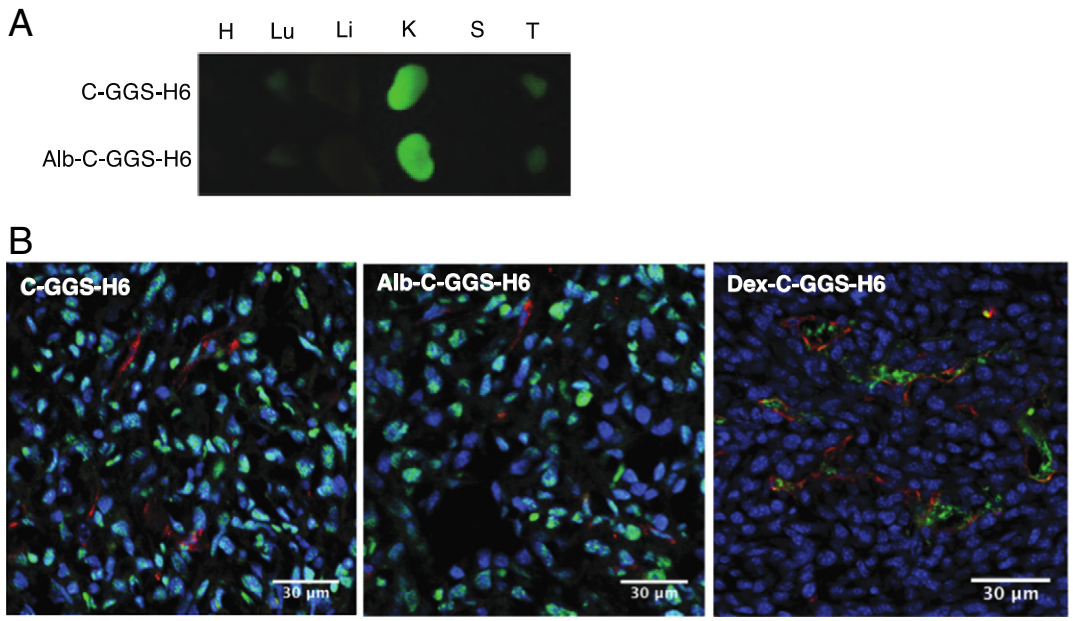

Fig. 5. Albumin conjugates exhibits similar tumor extravasation to free C-GGS-H6 peptide. (A) Representative images of peptide accumulation in tumors. C-GGS-H6 was pre-conjugated to mouse albumin *in vitro*, and the conjugates were intravenously injected into mice bearing 4T1 tumors (n = 3) and allowed to circulate for 1 h. After perfusion, major organs (H, heart; Lu, lung; Li, liver; K, kidney; S, spleen; T, tumor) were excised and their fluorescent signals were captured as described in the Materials and methods section. (B) Representative images of the distribution of C-GGS-H6, and its albumin and dextran conjugates in tumor tissues by immunofluorescence analysis. The peptide and its conjugates were detected using anti-His6 tag antibody (green). Blood vessels and nuclei were stained using rat anti-mouse CD31 antibody (red) and DAPI (blue).

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