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A tumor-penetrating peptide enhances circulation-independent targeting of peritoneal carcinomatosis

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Abstract

Peritoneal carcinomatosis is a major source of morbidity and mortality in patients with advanced abdominal neoplasms. Intraperitoneal chemotherapy (IPC) is an area of intense interest given its efficacy in ovarian cancer. However, IPC suffers from poor drug penetration into peritoneal tumors. As such, extensive cytoreductive surgery is required prior to IPC. Here, we explore the utility of iRGD, a tumor-penetrating peptide, for improved tumor-specific penetration of intraperitoneal compounds and enhanced IPC in mice. Intraperitoneally administered iRGD significantly enhanced penetration of an attached fluorescein into disseminated peritoneal tumor nodules. The penetration was tumor-specific, circulation-independent, and mediated by the neuropilin-binding RXXK tissue-penetration peptide motif of iRGD. Q-iRGD, which fluoresces upon cleavage, including the one that leads to RXXK activation, specifically labeled peritoneal metastases displaying different growth patterns in mice. Importantly, iRGD enhanced intratumoral entry of intraperitoneally co-injected dextran to approximately 300% and doxorubicin to 250%. Intraperitoneal iRGD/doxorubicin combination therapy inhibited the growth of bulky peritoneal tumors and reduced systemic drug toxicity. iRGD delivered attached fluorescein and co-applied nanoparticles deep into fresh human peritoneal metastasis explants. These results indicate that intraperitoneal iRGD co-administration serves as a simple and effective strategy to facilitate tumor detection and improve the therapeutic index of IPC for peritoneal carcinomatosis.

Graphical abstract

Keywords

peritoneal carcinomatosis; intraperitoneal chemotherapy; tumor-penetrating peptide; integrin; neuropilin

1. INTRODUCTION

Peritoneal metastases are present in up to 30% of colorectal, 50% of gastric, 65% of ovarian, and nearly 100% of appendiceal cancers at the time of diagnosis (1-4). The metastases cause complications that are not easily palliated, such as bowel obstruction and ascites. Systemic chemotherapy is less active against peritoneal metastasis than visceral metastasis. Tumor cell aggregates that float in ascites or have just attached to the peritoneum lack vasculature, preventing blood-borne drugs from reaching the tumor cells (5, 6). Systemically administered drugs partially enter the peritoneal fluid, but the concentrations achieved are not high enough to provide reliable anti-tumor effects (7). Advanced tumors with adequate blood flow have high interstitial fluid pressure, which inhibits intratumoral drug distribution (6, 8-11).

Intraperitoneal chemotherapy (IPC) overcomes the drug access issues in peritoneal metastasis treatment to some extent. Drug solution is administered into the abdominal cavity to bathe the tumors in a high concentration of the drug. However, reliable drug penetration into peritoneal tumors is limited to about 0.5 mm in depth (6, 11). Heating the drug solution improves drug penetration by causing vasodilation, breakage of tissue barriers, and increased drug diffusion into the tissue (1, 12, 13). However, drugs still do not enter tumors beyond a depth of 3-5 mm (11, 13). Thus, prior to IPC, tumors as small as 3 mm need to be removed by cytoreductive surgery for adequate therapeutic efficacy to be achieved.

Tumor-penetrating peptides are tumor-homing peptides that are actively transported through tumor tissue (14). The tumor-penetration activity of the peptides depends on the expression levels of three elements in the tumor tissue, primary receptor (e.g., integrins), proteases, and neuropilins. These peptides initially bind to a tumor-specific receptor such as integrins, and are then converted to binders of neuropilin-1 (NRP-1) and neuropilin-2 (NRP-2) through proteolytic cleavage that exposes a C-terminal RXXR/K amino acid sequence motif. The C-terminal location is a prerequisite for the NRP binding, and led to the naming of this motif as the C-end Rule or CendR motif (15).

A prototypic tumor-penetrating peptide, iRGD (cyclic CRGDK/RGPD/EC, cyclized between the two cysteines with a disulfide bond), carries a tumor-specific RGD motif, and an overlapping CendR motif (16). The RGD motif mediates iRGD binding to α_v integrins expressed on tumor endothelial cells and other cells in tumors, but not in normal tissues (17, 18). This initial integrin binding is required for the subsequent proteolytic step, which generates the N-terminal fragment of iRGD, CRGDK/R, and exposes a C-terminal CendR motif. Various proteases that cleave peptides after basic amino acid residues are highly expressed in tumor tissue (14, 19). The activated CendR motif binds to NRP-1 (and/or NRP-2), and initiates a transport system through the vascular wall and tumor tissue. The system uses a bulk transport mechanism based on a novel endocytosis/transcytosis pathway related to but distinct from macropinocytosis, which facilitates uptake of extracellular fluid containing bystander molecules (20). Thus, it transports compounds directly conjugated to the N-terminus of iRGD as well as compounds co-administered with iRGD (16, 21). Both iRGD conjugation and co-administration have been shown to enhance the therapeutic index of various intravenously administered anti-cancer compounds (16, 21-26). Interestingly, iRGD penetrates tumors not only through the vasculature, but also locally in a circulation-independent manner. Tumor cells often have high surface expression of both α_v integrins and NRP-1/NRP-2 (17, 27). Dipping freshly excised mouse tumors into iRGD solution allows iRGD and co-applied molecules to spread deep into the tumor tissue in a NRP-1- and energy-dependent manner (21). iRGD-tagged molecules injected directly into tumors spread widely within the tumor tissue, whereas molecules without iRGD spread minimally (28).

The ability of iRGD to enhance local penetration of co-applied molecules into tumor tissue prompted us to investigate the utility of iRGD for improving IPC. Here, we show that intraperitoneal (IP) iRGD delivers attached and co-administered molecules deep into peritoneal tumors regardless of the degree of tumor vascularity, and improves the therapeutic index of co-administered IPC in mice.

2. MATERIALS AND METHODS

2.1. Peptides

Fluorescein (FAM)-labeled iRGD (FAM-iRGD, cyclic FAM-Ahx-CRGDKGPDC with an amidated C-terminus; Ahx, aminohexanoic acid linker), FAM-CRGDC (cyclic FAM-Ahx-CRGDC), FAM-iRGDD (cyclic FAM-Ahx-CRGDDGPKC), FAM-ARA (linear FAM-Ahx-ARALPSQRSR with an amidated C-terminus) were synthesized as described (15, 16, 21). Quenched FAM-peptides (cyclic FAM-Ahx-peptide-K-Dabcyl) including Q-iRGD, Q-CRGDC, and Q-iRGDD, were purchased from LifeTein LLC (Hillsborough, NJ).

2.2. Cell lines and mouse models

Authenticated Lovo-6-luc-1 human colon cancer cells were purchased from Caliper Life Sciences (Hopkinton, MA). MKN45P human gastric cancer cells with high peritoneal dissemination potential was isolated from parental MKN45 cells and authenticated as described (29, 30). MKN45P-luc cells were made by infecting MKN45P cells with lenti-luciferase (Biogenova, Potomac, MD). Authenticated IGROV-1 human ovarian cancer cells (31) were a gift from Dr. Paola Perego (Istituto Nazionale Tumori, Milan). The cells were

used for no longer than 6 months before being replaced. For tumor mouse models, nude mice received IP injections of 1 million MKN45P or MKN45P-luc cells or 5 million Lovo-6-luc-1 or IGROV-1 cells. Animal experimentation procedures were approved by the Animal Research Committee at Sanford-Burnham Medical Research Institute.

2.3. Flow cytometry (16)

Cells were labeled with rabbit anti-human NRP-1 b1b2 (20), mouse antihuman $\alpha\beta 3$ (EMD Millipore, Billerica, MA), or mouse anti-human $\alpha\beta 5$ (EMD Millipore), followed by secondary antibodies with Alexa 488, 594 or 647 (Molecular Probes, Eugene, OR), and analyzed with an LSR Fortessa System (BD Biosciences, San Jose, CA). Analysis was done with Flowjo.

2.4. Peptide biodistribution

Peptides (0.3 mg in 0.5 ml of PBS) were intraperitoneally injected into tumor-bearing mice. After 1 hr, the mice were sacrificed under deep anesthesia and imaged under an Illuminatool Bright Light System LT-9900 (Lighttools Research, Encinitas, CA). Tissues were harvested, imaged under LT-9900, and processed for immunostaining. Circulation-dependent peptide homing to tumors was examined as described (16, 21). Briefly, 0.3 mg of peptide were injected into the tail vein of tumor mice. After 1 hr, the mice were perfused through the heart with PBS-1% BSA under deep anesthesia, and tissues were excised for *ex vivo* fluorescence imaging and immunofluorescence.

2.5. Immunofluorescence (16)

Frozen sections were stained with rat anti-mouse CD31 (BD Biosciences), rabbit anti-T7 phage (16), or rat anti-ER-TR7 (Abcam, Cambridge, MA) followed by secondary antibodies with Alexa 488 or 594. Human CD31 was stained with an Alexa 647 mouse anti-human CD31 (Biolegend, San Diego, CA). Images were taken with a Fluoview confocal microscope (Olympus, Center Valley, PA).

2.6. Immunocytochemistry (16)

Cells grown on collagen I-coated coverslips (BD Biosciences) were treated with 10 μ M peptide for 4 hr at 37°C. The cells were fixed, stained with DAPI (Molecular Probes), and viewed with a Fluoview confocal microscope. Some cells were treated with a blocking anti-NRP-1 b1b2 antibody or control IgG for 20 min before and throughout the peptide incubation.

2.7. *In vivo* bystander dextran penetration assay

Tumor mice were co-injected with 0.3 mg peptide and 0.3 mg Texas red-conjugated 3-kDa dextran (Molecular Probes) either into the abdominal cavity or tail vein. The volume given was 1 ml for IP and 0.2 ml for IV injections. After 90 min, the mice were sacrificed under deep anesthesia, and imaged under LT-9900 after brief rinsing of the abdominal cavity with PBS. Tissues were excised, imaged under LT-9900, and processed for immunofluorescence. The 90 min time point was chosen based on a clinical protocol for intraoperative IPC (12, 32).

2.8. *In vivo* iRGD/doxorubicin (DOX) combination delivery

Tumor-bearing mice received IP co-injections of 0.3 mg iRGD or PBS alone together with 4 mg/kg free DOX (Sigma-Aldrich, St. Louis, MO). One ml in total was given. After 90 min, the mice were sacrificed under deep anesthesia, and tissues were collected for DOX quantification following previous protocols (21, 33).

2.9. Long-term tumor treatment studies

Tumor cells were implanted intraperitoneally into mice 1 week (MKN45P-luc) or 3 weeks (Lovo-6-luc-1) prior to the initiation of the treatment study. The mice received IP injections of iRGD (14 μ mol/kg in 0.5 ml of PBS) or PBS alone followed 15 min later by IP injection of DOX (4 mg/kg in 0.5 ml of PBS) or PBS alone. The mice were treated every other day for 15 (MKN45P-luc) or 28 days (Lovo-6-luc-1), and imaged for bioluminescence every 4 (MKN45P-luc) or 7 (Lovo-6-luc-1) days using an IVIS imaging system (Xenogen, Alameda, CA). The mice were sacrificed under deep anesthesia, and tissues and body fluids were collected for further analysis.

2.10. *In vitro* cell toxicity assay

MKN45P-luc or Lovo-6-luc-1 cells were seeded in 96-well culture plates at 2×10^4 cells per well and incubated overnight. The cells were incubated with DOX (100 μ M) for 90 min at 37°C in the presence or absence of FAM-iRGD (50 μ M) or an irrelevant control peptide (FAM-ARALPSQRSR, 50 μ M). Some wells were treated with iRGD or PBS alone. The cells were washed, cultured in fresh media for 50 hr, and viability was assessed by MTT assays (Thermo Fisher Scientific, Waltham, MA) (16).

2.11. *Ex vivo* tumor dipping assay

This assay allows evaluation of compound penetration into human tissues without having to inject the compounds into human patients (21, 34). Fresh surgical samples of peritoneal metastases of colon, appendiceal, and ovarian cancer and malignant mesothelioma were collected under protocols approved by an Institutional Review Board at the University of California San Diego and Columbia University. The samples were immediately cultured in media containing 10 μ M of peptide and 10^9 plaque-forming units (pfu) of phage for 90 min at 37°C. The tumors were processed for immunofluorescence after extensive washes with PBS.

2.12. Statistical analysis

Student's *t*-test or one-way analysis of variance (ANOVA) was performed with Prism v6.0e (GraphPad Software, La Jolla, CA). Survival curves were analyzed with log-rank test. Results are summarized in Supplementary Table S1.

3. RESULTS

3.1. iRGD delivers an attached chemical into peritoneal metastases through the abdominal cavity

We used a peritoneal carcinomatosis mouse model created by IP injection of MKN45P cells, an aggressive subpopulation of MKN45 human gastric cancer cells that form disseminated peritoneal tumors (34). The MKN45P cells express cell surface integrin and NRP-1, the iRGD receptors (Supplementary Fig. S1a). MKN45P peritoneal tumors grown in mice express α_v integrins and NRP-1 at high levels, while normal organs such as the liver express lower levels of these proteins (Supplementary Fig. S1b). IP injection of FAM-iRGD into MKN45P tumor-bearing mice led to effective FAM entry into peritoneal metastases with a thickness ranging from a few mm up to 10 mm (Fig. 1a). CRGDC is a control RGD peptide without a CendR motif, which interacts with α_v integrins with a similar affinity as iRGD but lacks affinity to NRP-1 (16). FAM-CRGDC only weakly labeled tumor surfaces demonstrating the critical roles of CendR in the iRGD activities. iRGD delivered FAM into the tumors approximately 500% more efficiently than CRGDC without affecting normal organs, suggesting the tumor-specific penetration activities of iRGD (Fig. 1b). Immunofluorescence confirmed the tumor specificity of the iRGD-mediated FAM delivery: FAM signals were present deep inside peritoneal tumor nodules, but not in adjacent normal organs (Fig. 1c, left panel). Reactive fibrosis surrounding the tumor nodules (35, 36) was often positive for FAM-iRGD (Fig. 1c, left panel, arrowheads). The fibrotic structure consisted of reticular fibroblasts and fibers, and was strongly positive for α_v integrins and NRP-1 (Supplementary Fig. S2). In contrast, CRGDC only delivered FAM to peritoneal tumor surfaces with less tumor specificity (Fig. 1c, right panel). FAM-CRGDC distributed within tumor cell aggregates consisting of a few tumor cell layers, but failed to penetrate larger nodules, in agreement with earlier findings that IP drugs only penetrate into peritoneal tumors up to 0.5 mm (Fig. 1c, right panel, inset) (11). Similar results were obtained in mice with disseminated peritoneal metastases created with Lovo-6-luc-1 human colon and IGROV-1 human ovarian cancer cells both of which express cell surface α_v integrins and NRP-1 (Supplementary Fig. S1, S3, S4).

3.2. IP iRGD targets peritoneal metastases in a circulation-independent manner

Compounds injected into the abdominal cavity enter systemic circulation following absorption from the peritoneal cavity (37, 38). Thus, the peritoneal tumor targeting achieved by IP FAM-iRGD might have been a result of tumor penetration from the vascular compartment. To this end, we examined whether IP FAM-iRGD targets extraperitoneal tumors. IP iRGD delivered FAM efficiently into peritoneal tumor nodules, but minimally into subcutaneous tumors (Fig. 2a). FAM accumulation in subcutaneous tumors was approximately 25% of that in peritoneal tumors suggesting that up to one-fourth of the peritoneal tumor targeting was accomplished by circulating iRGD (Fig. 2b). In contrast, intravenous (IV) FAM-iRGD accumulated equally in subcutaneous and peritoneal tumors likely because of the large amount of angiogenic vasculature in the tumors regardless of the location. IP delivery was significantly more effective in targeting peritoneal metastases than IV delivery in line with the advantages of IPC over systemic chemotherapy for peritoneal

tumors (5, 6). FAM accumulation into normal organs was minimal regardless of the injection route, again demonstrating the specificity of iRGD for malignant tissue (16).

Immunofluorescence of tumors collected from mice that received IP injection of FAM-iRGD revealed extensive FAM distribution within peritoneal tumors and minimal FAM signals in subcutaneous tumors (Fig. 2c). In contrast, IV injection of FAM-iRGD resulted in widespread FAM signals in both peritoneal and subcutaneous tumors. Importantly, IP FAM-iRGD accumulated into both small avascular peritoneal nodules and large vascularized peritoneal tumors, while IV FAM-iRGD only targeted vascularized tumors. These results indicate that IP injection of iRGD leads to peritoneal tumor targeting mainly through locoregional penetration, and that only a minor contribution of circulating iRGD was involved.

3.3. iRGD is proteolytically processed during peritoneal tumor cell penetration

iRGD triggers a transtumoral active bulk transport system by interacting with NRP-1 using its RXXK CendR peptide motif (16). The CendR motif is inactive unless it is exposed at the C-terminus by proteolytic processing (15). To image the CendR activation during peritoneal tumor cell targeting, we designed Q-iRGD, a modified FAM-iRGD peptide that fluoresces in green upon proteolytic processing. Q-iRGD [amino acid sequence: FAM-CRGDKGPDC-K(Dabcyl)] carries an N-terminal FAM and a C-terminal lysine conjugated to a Dabcyl quencher. The two cysteines are disulfide bonded, and the C-terminus has a free carboxyl. The Dabcyl group quenches the FAM by ~90% owing to the cyclic peptide structure. Linearization of Q-iRGD by reduction of the disulfide bond only marginally decreases quenching (~86%) making proteolytic processing an essential step for Q-iRGD to effectively fluoresce in green. Cultured MKN45P, Lovo-6-luc-1, and IGROV-1 cells extensively fluoresced when incubated with Q-iRGD indicating the presence of cleaved Q-iRGD within the cells (Supplementary Fig. S5). The fluorescence was reduced when the cells were treated with a blocking anti-NRP-1 b1b2 antibody, which inhibits CendR-NRP-1 interaction, indicating that the cleavage occurred before peptide uptake. Q-CRGDC and Q-iRGDD, quenched control RGD peptides that lack a CendR motif, yielded minimal fluorescence supporting the role of CendR-NRP-1 interaction in Q-iRGD internalization. These results indicate that cellular uptake of Q-iRGD involves proteolytic activation and subsequent interaction with NRP-1.

3.4. Q-iRGD as a reporter for *in vivo* activation of the CendR tumor-penetration pathway

IP injection of Q-iRGD into mice bearing IGROV-1 peritoneal tumors resulted in extensive fluorescence tumor labeling, facilitating detection of disseminated peritoneal tumors (Fig. 3, upper panels). Normal organs were minimally affected (except for intestine and kidneys from which peptides are excreted), suggesting that the proteolytic activation of iRGD occurred after tumor targeting and not randomly in the abdominal cavity. IP injection of Q-CRGDC and Q-iRGDD led to minimal green fluorescence in the tumors indicating the CendR dependence of the iRGD activity (Supplementary Fig. S6a). Fluorescence intensity in the peritoneal tumors was approximately 300% higher in the Q-iRGD injected group than the Q-CRGDC or Q-iRGDD control group (Supplementary Fig. S6b). Fluorescence in normal tissues was comparable among all groups. When compared to FAM-iRGD, Q-iRGD

provided similar fluorescence intensity in peritoneal tumors but less fluorescence in most of the normal abdominal tissues analyzed, suggesting the high specificity of Q-iRGD in labeling peritoneal tumors (Supplementary Fig. S6c). Immunofluorescence confirmed the presence of fluorescent (dequenched) Q-iRGD within the peritoneal tumors but not in normal organs, and the minimal presence of fluorescent Q-CRGDC and Q-iRGDD in the tumors (Fig. 3, right upper panel; Supplementary Fig. S6a, right panels).

In mice bearing early-stage Lovo-6-luc-1 peritoneal tumors, intraperitoneally administered Q-iRGD produced green fluorescence in disseminated small avascular nodules, which were otherwise difficult to visualize without fluorescence imaging (Fig. 3, middle panels). Abdominal neoplasms sometimes spread as scirrhous sheets on peritoneal surfaces necessitating extensive peritonectomy (32). Q-iRGD labeled metastatic sheets of MKN45P on the surface of the diaphragm, as well as nodular peritoneal tumors (Fig. 3, lower panels). However, a subcutaneous tumor did not fluoresce in green suggesting circulation-independent targeting. These data indicate the capacity of Q-iRGD to label various growth patterns of peritoneal tumors through local penetration.

3.5. iRGD facilitates co-injected IP dextran accumulation into peritoneal tumors

An important feature of iRGD is its ability to deliver systemically co-injected bystander free molecules into tumor tissue (21). The delivery uses a bulk transport mechanism, which is activated through the vasculature and also within the tumor tissue (21). Thus, we hypothesized that IP iRGD delivers bystander free molecules into peritoneal tumors through local penetration. The hypothesis was tested in mice bearing Lovo-6-luc-1 peritoneal tumors using Texas red-labeled dextran as a bystander tracer. IP iRGD enhanced dextran entry into peritoneal tumors as thick as 10 mm, while CRGDC had a negligible effect (Fig. 4a). iRGD tripled intratumoral dextran accumulation in a tumor-specific manner (Fig. 4b).

Immunofluorescence showed widespread dextran signals in both avascular and vascularized peritoneal tumors with iRGD IP co-injection, suggesting that the dextran penetration was circulation independent (Fig. 4c, left two panels). Peritoneal tumors from mice that received IP CRGDC and dextran showed dextran signals on the tumor surface but minimally inside the tumor tissue (Fig. 4c, right most panel). Similar results were obtained in mice bearing IGROV-1 peritoneal tumors (Supplementary Fig. S7).

3.6. IP iRGD delivers co-administered dextran into peritoneal tumors by local penetration

IP iRGD also delivered co-injected dextran into MKN45P peritoneal tumors, but not into subcutaneous tumors (Fig. 5a, left panel). IV co-injection of the two agents led to dextran entry into both peritoneal and subcutaneous tumors (Fig. 5a, right panel). Both IP and IV iRGD co-injection approximately tripled dextran levels in peritoneal tumors in a tumor-specific manner (Fig. 5b). However, the actual amount of dextran in peritoneal tumors was higher in the IP group than the IV group (Fig. 5c), likely because a higher local dextran concentration around the peritoneal tumors was achieved with IP injection.

Confocal microscopy showed widespread dextran accumulation in avascular and vascularized peritoneal tumors collected from mice that received IP co-injection of iRGD (Fig. 5d, left panels). Minimal dextran signals were observed in subcutaneous tumors. IV co-

injection of iRGD facilitated dextran distribution within vascularized peritoneal and subcutaneous tumors, but not in poorly vascularized peritoneal tumors (Fig. 5d, right panels). IP or IV injection of dextran alone led to minimal dextran accumulation into peritoneal and subcutaneous tumors (Supplementary Fig. S8). These results indicate that IP iRGD facilitates entry of bystander compounds into peritoneal tumors mainly by local penetration.

3.7. iRGD improves the therapeutic index of co-administered IPC in peritoneal tumor mouse models

The bystander effect of IP iRGD suggested its utility in potentiating IPC. We tested the effect of iRGD IP co-injection on intratumoral delivery and anti-tumor efficacy of DOX, a drug used in the treatment of gastrointestinal neoplasms including gastric cancer (39). IP iRGD doubled the entry of free DOX into peritoneal tumors created with luciferase-expressing MKN45P (MKN45P-luc) cells (Fig. 6a). Although not statistically significant, iRGD showed a trend to decrease DOX entry in normal organs.

Bioluminescent live animal imaging was performed to measure tumor burden during long-term IPC in mice bearing MKN45P-luc-derived peritoneal tumors. This revealed a trend toward a lower burden of disease in mice treated with iRGD/DOX combination as compared to DOX alone. (Supplementary Fig. S9a). However, iRGD/DOX combination therapy was significantly more effective than DOX alone in inhibiting the growth of the omental cake, a large tumor bulk formed in the greater omentum (Fig. 6b) (40). The effect of iRGD in enhancing DOX activity against small peritoneal tumor nodules (1-3 mm in diameter; see Supplementary Fig. S9b) was not significant likely because DOX penetration was sufficient to be active against small nodules (Fig. 6c). Of note, iRGD alone did not increase the number of peritoneal tumor nodules suggesting that iRGD does not increase the risk of further peritoneal metastasis. Serial measurement of mouse body weight showed that iRGD IP co-injection did not increase systemic DOX toxicity at the dose used in this study (Supplementary Fig. S9c). The small increase in body weight at the last time point was likely secondary to ascites accumulation (Supplementary Fig. S9d). These results indicate that iRGD co-injection potentiates IPC mainly against large peritoneal tumors without increasing systemic toxicity.

The bystander effect of iRGD was observed not only at tissue levels but also at cellular levels. *In vitro* cell toxicity assays revealed that MKN45P-luc cells were resistant to DOX at baseline (Supplementary Fig. S10a). Addition of free iRGD peptide in the culture media made MKN45P cells significantly more sensitive to DOX. These results are consistent with earlier findings that iRGD facilitates internalization of bystander molecules into tumor cells through a macropinocytosis-like mechanism (20) and that MKN45P-luc tumors kept growing *in vivo* despite DOX treatment.

IP iRGD increased co-injected DOX accumulation into Lovo-6-luc-1 peritoneal tumors to 250% (Fig. 6d). Interestingly, iRGD co-injection significantly reduced DOX accumulation into the spleen (Supplementary Fig. 11a). A similar trend was observed in other abdominal organs such as the liver and kidney. Long-term iRGD/DOX combination therapy in mice with Lovo-6-luc-1-derived peritoneal tumors significantly inhibited tumor growth, although

the effect was not significant when compared with DOX alone (Fig. 6e), likely because the Lovo-6-luc-1 cells are highly sensitive to DOX (Supplementary Fig. S11b). In fact, iRGD did not potentiate DOX in cultured Lovo-6-luc-1 cells either. However, just as importantly, iRGD significantly reduced the systemic toxicity of DOX. Seventy-five percent of mice treated with DOX required early termination secondary to systemic toxicity, while only 12.5% of mice in the iRGD/DOX treatment group required early termination (Supplementary Fig. S11b-d). This finding is in agreement with the reduced DOX concentrations in normal organs upon iRGD co-injection.

These results indicate that iRGD can either potentiate IPC especially in bulky tumors, reduce systemic toxicity of an IP drug without decreasing the anti-tumor effects, or possibly be used to balance both.

3.8. iRGD and co-applied phage nanoparticles penetrate human peritoneal metastasis explants

To investigate the relevance of the iRGD IP drug delivery system in humans, we tested whether iRGD facilitates penetration of attached FAM and co-applied inert phage nanoparticles (65 nm in diameter) into freshly excised human peritoneal tumor explants in tumor-dipping assays (21, 32). Fresh peritoneal metastases were collected from appendiceal, colon, and ovarian cancer patients during surgical cytoreduction. FAM and co-applied phage efficiently penetrated into all of the human tumors tested (Fig. 7). Surprisingly, FAM-iRGD completely penetrated tumors thicker than 30 mm, suggesting the utility of iRGD in delivering drugs into large peritoneal tumors, which currently require surgical removal prior to IPC. FAM-CRGDC or FAM-iRGDD and co-applied phage failed to penetrate the tumors. Similar to the MKN45P, Lovo-6-luc-1, and IGROV-1 xenograft tumors in mice, human peritoneal tumors were rich in angiogenic blood vessels and fibrotic stroma (Supplementary Fig. S12). These findings suggest that the iRGD IP drug delivery system may function in humans.

4. Discussion

This study introduces an innovative application of a prototypic tumor-penetrating iRGD peptide for enhancement of IP drug delivery. We demonstrate that IP injection of the peptide allows selective delivery of compounds attached to and co-administered with the iRGD peptide into peritoneal tumors in a manner independent of blood circulation.

iRGD facilitated 500% greater accumulation of its conjugated cargo (FAM) into peritoneal tumors than CRGDC, an RGD control peptide, which binds to α_v integrins with a similar affinity as iRGD but lacks affinity to the tissue-penetrating neuropilin receptors (16). The poor tumor penetration of FAM-CRGDC even into small tumor cell aggregates agrees with the fact that IP molecules have limited tumor penetration (11, 13). The tumor penetrating activities of iRGD provide an opportunity to accomplish conjugation-based intraperitoneal delivery of imaging agents and drugs deep into peritoneal tumors.

IP delivery has several advantages over systemic delivery when targeting iRGD-bound compounds to peritoneal tumors. First, IP delivery achieves substantially more accumulation

of compounds (FAM in this study) into peritoneal tumors than IV delivery. Second, IP delivery has significantly less influence on extraperitoneal organs than IV delivery, given that IP injection of FAM-iRGD resulted in minimal FAM accumulation into subcutaneous tumors. Third, IP iRGD delivered FAM into peritoneal tumors with various sizes regardless of vascularity, while IV delivery did not lead to detectable targeting of non-vascularized peritoneal tumor nodules. Earlier results on the ability of iRGD to improve tumor treatment suggested that similar improvements could be achieved with disseminated peritoneal tumors, and expectation borne out by the present results (21-23).

Q-iRGD, which highlights the prerequisite proteolytic activation step for iRGD, showed promise as a tumor-guidance probe to stratify patients for iRGD-based IPC. IP Q-iRGD labeled various types of peritoneal tumors regardless of their growth patterns with high sensitivity and specificity. The dequenched Q-iRGD accumulation into peritoneal tumors was caused by CendR-dependent penetration and not by integrin-mediated uptake of the intact peptide or non-specific diffusion of randomly proteolysed peptide fragments, considering the limited fluorescence provided by control peptides.

An important finding was that IP injection of iRGD facilitated accumulation of bystander compounds into peritoneal tumors. Similar to attached FAM delivery, the accumulation was mainly achieved through local tumor penetration demonstrated by prominent dextran signals even in non-vascularized nodules and minimal signals in extraperitoneal tumors. In contrast, IV co-injection limited the targeting to vascular-rich tumors. Thus, IP delivery appears to be the preferred route for peritoneal tumor targeting with iRGD co-injection. Another reason to choose IP over IV injection for peritoneal metastasis targeting with iRGD combination delivery is that IP injection leads to higher intratumoral drug accumulation. iRGD combination delivery relies on a CendR-mediated transtumoral bulk transport system, the efficacy of which depends on the local concentration of the bystander drug (20, 21). Unlike IV injection, IP injection does not result in immediate drug dilution by blood (unless there is significant ascites), providing the opportunity to achieve higher local drug concentration around the tumors. In the presence of ascites, a one-time IP perfusion chemotherapy performed during cytoreductive surgery can eliminate the dilution effect of ascites (12, 32). The result was a 50% higher intratumoral drug accumulation than with IV injection in this study.

iRGD combination IPC appears to be particularly effective in inhibiting the growth of large peritoneal tumors into which IP drugs alone minimally penetrate (6, 11). Targeting of locally advanced tumors that may involve critical structures such as major blood vessels and intestine would significantly decrease the complexity of cytoreduction and increase the number of patients who could be treated in this manner. Given that FAM-iRGD and dextran co-injected with iRGD penetrated 10 mm-thick mouse peritoneal tumors *in vivo*, it is evident that iRGD has the capacity to deliver molecules as deep as 5 mm. However, the *ex vivo* tumor-dipping assay suggests that iRGD may achieve even deeper penetration of larger molecules up to a nano scale: iRGD delivered an attached FAM and co-injected phage (diameter: 65 nm) into human tumors thicker than 30 mm. With further optimization of administration schedules, iRGD may accomplish drug delivery into substantially large tumors to reduce or eliminate the necessity of cytoreductive surgery, a major source of

morbidity associated with peritoneal carcinomatosis treatment. In fact, using higher drug doses seems possible given that iRGD minimally affects normal organs and may even reduce drug toxicity to the normal organs in some cases. The decreased drug uptake in non-cancerous tissue with iRGD co-administration may be explained by less drug availability secondary to significantly increased drug uptake into peritoneal tumors. iRGD may also change the role of hyperthermia in IPC if it helps achieve a similar or higher degree of drug penetration than hyperthermia, or if iRGD and hyperthermia have additive/synergistic effects on intratumoral drug penetration. Importantly, iRGD does not appear to facilitate further peritoneal metastasis unlike some of the enzymatic drug delivery approaches that dissociate tumors (41, 42). In fact, iRGD may even be protective against peritoneal metastasis since it suppresses tumor cell motility as a chemorepellent (43).

For clinical translation, the efficacy and toxicity of the iRGD system need to be evaluated under conditions that mimic clinical protocols, and in human trials. However, our data in human tumor explants strongly suggest that metastases from appendiceal, colon, and ovarian cancer and malignant mesothelioma carry all the elements required for iRGD to be functional in a human system. The data also indicate that using FAM-iRGD (or other forms of imageable iRGD peptide probes) for combination delivery would provide a way to accomplish theranostics, tumor detection and drug delivery to tumors at once (44). iRGD may significantly simplify the treatment of peritoneal carcinomatosis by providing accuracy in tumor detection and enhanced drug penetration into peritoneal tumors. The result will be a significant improvement in therapeutic outcome and patients' quality of life.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

IPC	intraperitoneal chemotherapy
NRP-1	neuropilin-1
NRP-2	neuropilin-2

single-letter abbreviations for amino acid residues	(A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; Y, Tyr; and X, any amino acid)
CendR	C-end Rule
IP	intraperitoneal
FAM	fluorescein
Ahx	aminohexanoic acid linker
PBS	phosphate buffered saline
DOX	doxorubicin
ANOVA	analysis of variance
IV	intravenous
Q-peptide (e.g., Q-iRGD)	quenched FAM-peptide

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Fig. 1. Peritoneal tumor targeting by iRGD through the abdominal cavity

Mice bearing disseminated MKN45P human gastric cancer peritoneal tumors received IP injections of FAM-iRGD or FAM-CRGDC. (a) Results of *in vivo* fluorescence imaging of necropsied mice and *ex vivo* fluorescence imaging of resected mouse tissues. Representative images of three independent experiments are shown. (b) Fluorescence intensity from the *ex vivo* imaging was quantified with Image J and normalized against the tissue area. Statistical analysis, Student's *t*-test; *n* = 3 per group; error bars, mean + SEM; ns, not significant; ***P* < 0.01. (c) Confocal microscopy images of peritoneal tumor sections. The arrowheads in the left panel show a fibrotic structure around a peritoneal tumor. The inset in the right panel shows avascular tumor cell aggregates. Note that FAM entered only the smaller aggregate on the right. Green, peptide; red, CD31; blue, DAPI; scale bars, 100 μ m. Black/white dashed lines, peritoneal tumors (PT); gray dashed lines, normal organs; Ht, heart; Lg, lung; Lv, liver; Sp, spleen; Pa, pancreas; Om, omentum; Kd, kidney; St, stomach.

Fig. 3. Fluorescence detection of disseminated peritoneal tumors with a quenched iRGD probe
Mice bearing disseminated peritoneal tumors of IGROV-1 ovarian, Lovo-6-luc-1 colon, and MKN45P gastric cancer received IP injections of a quenched FAM-iRGD probe, Q-iRGD, which highlights the prerequisite proteolytic activation step for iRGD. Results of *in vivo* fluorescence imaging of the mice, *ex vivo* fluorescence imaging of resected tissues, and confocal micrographs of tumor sections are shown. Green, unquenched peptide; red, CD31; blue, DAPI; scale bars, 100 μ m. Note that Q-iRGD facilitated detection of bulky peritoneal tumors (white dashed lines), small peritoneal tumor nodules (arrowheads), and metastatic sheets of MKN45P (blue dashed line) on the diaphragm (D). Yellow dashed lines, subcutaneous tumor (SCT); gray dashed lines, normal organs. SCT appears green medially because of the bright peritoneal tumor. Representative images of three independent experiments are shown. Ht, heart; Lg, lung; Lv, liver; Sp, spleen; Pa, pancreas; Om, omentum; Kd, kidney; St, stomach; Du, duodenum; Int, intestine.

Fig. 4. Dextran entry into peritoneal tumors facilitated by iRGD IP co-injection

Mice bearing peritoneal tumors of Lovo-6-luc-1 human colon cancer cells received IP co-injection of Texas red dextran and iRGD, CRGDC, or PBS. (a) *In vivo* fluorescence imaging of necropsied mice and *ex vivo* fluorescence imaging of resected tissues. Representative images of three experiments are shown. (b) Fluorescence intensity from the *ex vivo* imaging quantified with Image J and normalized to the tissue area. The results are shown as fold over dextran injection alone. Statistics, Student's *t*-test; *n* = 3 per group; error bars, mean + SEM; ns, not significant; ****P* < 0.001. (c) Confocal micrographs. Green, CD31; red, dextran; blue, DAPI; scale bars, 100 μ m. Black/white dashed lines, peritoneal tumors (PT); gray dashed lines, normal organs; Ht, heart; Lg, lung; Lv, liver; Sp, spleen; Pa, pancreas; Om, omentum; Kd, kidney; St, stomach.

Fig. 5. Circulation-independent dextran entry into peritoneal tumors facilitated by iRGD IP co-injection

Mice bearing disseminated peritoneal tumors created with Lovo-6-luc-1 human colon cancer cells received IP or IV co-injection of Texas red 3-kDa dextran and iRGD. (a) *Ex vivo* fluorescence imaging of mouse tissues. Representative images of three experiments are shown. (b, c) Fluorescence intensity from the *ex vivo* imaging quantified with Image J. Results are shown as fold over dextran injection alone (b) or after normalization to the tissue area (c). Statistics, Student's *t*-test; *n* = 3 per group; error bars, mean + SEM; ns, not significant; **P* < 0.05; ***P* < 0.01. (d) Confocal micrographs. The arrowhead points to a small tumor cell aggregate growing on the omentum. Green, CD31; red, dextran; blue, DAPI; scale bars, 100 μ m. White dashed lines, peritoneal tumors (PT); yellow dashed lines, subcutaneous tumors (SCT); gray dashed lines, normal organs. Ht, heart; Lg, lung; Lv, liver; Sp, spleen; Pa, pancreas; Om, omentum; Kd, kidney.

Fig. 6. Increased therapeutic index of IP DOX co-injected with iRGD

(a, d) Mice bearing disseminated peritoneal tumors created with MKN45P-luc human gastric (a) or Lovo-6-luc-1 human colon (d) cancer cells received IP co-injections of DOX (4 mg/kg) and 0.3 mg of iRGD or PBS alone. DOX in the tissues was quantified 90 min later. $n = 3$ per group. (b, c, e) Mice bearing disseminated MKN45P-luc (b, c) or Lovo-6-luc-1 (e) peritoneal tumors implanted 1 week (MKN45P-luc) or 3 weeks (Lovo-6-luc-1) earlier received IP injections of iRGD (4 μmol/kg) or PBS followed by DOX (4 mg/kg) or PBS, every other day. Tumors were harvested after 15 (MKN45P) or 28 days (Lovo-6-luc-1). Large omental tumors were weighed (b, e), and the numbers of remaining small tumor nodules (1-3 mm in general) were counted (c). $n = 5$ (MKN45P) and $n = 8$ (Lovo-6-luc-1) per group. Statistical analyses, Student's *t*-test (a, d) and ANOVA (b, c, e); error bars, mean + SEM; ns, not significant; * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

Fig. 7. Co-penetration of iRGD and phage nanoparticles into human peritoneal metastases

Fresh surgical explants of peritoneal metastases sampled from human appendiceal, colon, and ovarian cancer patients were cultured for 90 min at 37°C in the presence of FAM-peptides (green) and 10^9 pfu/ml of inert phage particles expressing G₇ peptides (G₇ phage, red). Confocal micrographs of the tumors are shown. Blue, DAPI; scale bars, 100 μ m. Note that FAM and phage widely distributed within the tumors with iRGD, but not with CRGDC, a non-CendR RGD peptide, or iRGDD (FAM-CRGDDGPKC), a scrambled iRGD peptide with a disrupted CendR motif. Representative images of five (appendiceal cancer) and three (colon and ovarian cancer) independent experiments are shown.