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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.

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].

Abbreviations: IPC, intraperitoneal chemotherapy; NRP-1, neuropilin-1; NRP-2, neuropilin-2; 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; 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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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 αv 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 αv 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 (Instituto 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 anti-human αvβ3 (EMD Millipore, Billerica, MA), or mouse anti-human αvβ5 (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 h, the mice were sacrificed under deep anesthesia and imaged under an Illuminatool Bright Light System LT-9900 (Lightools 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 was injected into the tail vein of tumor mice. After 1 h, 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 μM peptide for 4 h 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].