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A genetic fiber modification to achieve matrix-metalloprotease-activated infectivity of oncolytic adenovirus

Anabel José [a,b,1], Maria Rovira-Rigau [a,b,1], Jeroni Luna [a,b], Marta Giménez-Alejandre [f], Eva Vaquero [a,c,d], Beatriz García de la Torre [e], David Andreu [e], Ramon Alemany [f], Cristina Fillat [a,b,*]

[a] Institut d'Investigacions Biomèdiques August Pi iSunyer (IDIBAPS), Barcelona, Spain
[b] Centro de Investigación Biomédica en Red de Enfermedades Raras (CIBERER), Barcelona, Spain
[c] Department of Gastroenterology, Institut de Malalties Digestives i Metabòliques, Hospital Clínic, Barcelona, Spain
[d] Centro de Investigación Biomédica en Red de enfermedades hepáticas y digestivas (CIBERehd), Barcelona, Spain
[e] Universitat Pompeu Fabra, Barcelona, Spain
[f] Laboratori de Recerca Traslacional IDIBELL, Institut Català d'Oncologia, L'Hospitalet de Llobregat, Spain

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ABSTRACT

Selective tumor targeting of oncolytic adenovirus at the level of cell entry remains a major challenge to improve efficacy and safety. Matrix metalloproteases (MMPs) are overexpressed in a variety of tumors and in particular in pancreatic cancer. In the current work, we have exploited the expression of MMPs together with the penetration capabilities of a TAT-like peptide to engineer tumor selective adenoviruses. We have generated adenoviruses containing CAR-binding ablated fibers further modified with a C-terminus TAT-like peptide linked to a blocking domain by an MMP-cleavable sequence. This linker resulted in a MMP-dependent cell transduction of the reporter MMP-activatable virus AdTATMMP and in efficient transduction of neoplastic cells and cancer-associated fibroblasts. Intravenous and intraductal administration of AdTATMMP into mice showed very low AdTATMMP activity in the normal pancreas, whereas increased transduction was observed in pancreatic tumors of transgenic Ela-myc mice. Intraductal administration of AdTATMMP into mice bearing orthotopic tumors led to a 25-fold increase in tumor targeting compared to the wild type fiber control. A replication competent adenovirus, Ad^{RC}MMP, with the MMP-activatable fiber showed oncolytic efficacy and increased antitumor activity compared to Adwt in a pancreatic orthotopic model. Reduced local and distant metastases were observed in Ad^{RC}MMP treated-mice. Moreover, no signs of pancreatic toxicity were detected. We conclude that MMP-activatable adenovirus may be beneficial for pancreatic cancer treatment.

1. Introduction

Adenovirus serotype 5 (Ad5) is commonly used in the development of oncolytic agents for cancer gene therapy. Ad5 infectivity depends on the expression of the coxsackievirus–adenovirus-receptor (CAR) on the surface of target cells. However, since CAR is frequently downregulated in tumor cells, Ad5 neoplastic cell transduction is often inefficient, limiting the anti-cancer effects of oncolytic adenoviruses. Hence, efforts to improve adenoviral tumor cell transduction have been developed. Strategies such as fiber pseudotyping [1], the addition of high affinity ligands to different adenovirus capsid proteins [2–5], and the incorporation of chemically or conjugated surface modifications have been studied [6–8]. In particular, the protein transduction domain Tat-PTD from the HIV-1 Tat protein has been inserted into the HI Loop or the C-terminus of the fiber knob [3] and into the hexon hypervariable region 5 (HVR5) of the virus capsid showing increased transduction efficacy on a variety of tumor cells [5].

An optimal retargeted virus should combine an efficient tumor cell transduction with tumor selectivity. Viral activation through cancer-specific proteases is an approach that has been tested to redirect enveloped viruses to cancer cells. Since most enveloped viruses require protease cleavage of viral glycoproteins for productive cell entry, retroviral glycoproteins have been engineered with matrix metalloproteases (MMP)-cleavable linkers to provide retroviruses with cancer specificity [9,10]. Measles and Sendai viruses have also been modified with similar strategies showing tumor selectivity [11,12].

In the current work we studied the potential to confine adenoviral oncolysis to tumor tissue with the dual objective to: i) improve adenoviral tumor transduction and ii) achieve adenoviral specific activation in tumor cells. We have generated a fiber-modified adenovirus with a TAT-like domain exposed upon cleavage with tumor-specific proteases. We show MMP-dependent selectivity of the virus, increased tumor

* Corresponding author at: Institut d'Investigacions Biomèdiques August Pi iSunyer-IDIBAPS, Rosselló 149-153, 08036-Barcelona, Spain. Tel.: +34 932275400x4579.
E-mail address: cfillat@clinic.ub.es (C. Fillat).
[1] Anabel José and Maria Rovira-Rigau equally contributed to the work.

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targeting and remarkable oncolytic antitumoral efficacy in pancreatic xenografts.

2. Materials and methods

2.1. Cell lines

HEK293, NIH-3T3 cells, and the human pancreatic adenocarcinoma cell line PANC-1 were obtained from the American Type Cancer Collection (ATCC). The human pancreatic adenocarcinoma cell line RWP-1 and the HT1080 fibrosarcoma cell line were kindly provided by Dr F.X. Real and Dr. R. Alemany, respectively. Cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and antibiotics, and cultured as described [13]. PSC-21, CAF-25, and CAF-28 cells were isolated from fresh pancreatic specimens obtained from adult patients undergoing surgical resection for pancreatic cancer at the Hospital Clinic in Barcelona. All human samples were obtained under the approval of the Institutional Ethics Committee. Tissue blocks were cut into small pieces and dissociated in collagenase solution. Digested tissue was filtered and later centrifuged on a 12% Optiprep density gradient. A resultant fuzzy band at the interface and the cell pellet were collected separately, suspended in DMEM/F-12 medium, and cultured with DMEM-F-12 medium containing 15% FBS, Glutamax, antibiotics, HEPES, insulin, and IGF-1. Fibroblasts collected from the interface were identified as quiescent pancreatic stellate cells (PSCs) and fibroblasts growing out from pellets were considered as cancer associated fibroblasts (CAFs). Activated PSCs and CAFs were characterized by immunostaining. Both cell types showed abundant expression of α-smooth muscle actin and vimentin and were negative for E-cadherin.

2.2. Adenovirus construction

AdCMVGFPLuc (AdTL) has been previously described [14]. Adwt, the wild-type adenovirus, was obtained from the ATCC (Manassas, VA). In the present work we have generated the recombinant adenoviruses AdYTRGE, AdTATMMP, and the replication-competent virus Ad^{RC}MMP. AdTL, AdYTRGE, and AdTATMMP express the enhanced GFP gene and the firefly luciferase gene under the control of a CMV promoter.

The AdYTRGE was generated modifying the plasmid backbone of pAdTL. Three modifications were performed on the fiber gene: i) the Y477A mutation (TAT changed to GCT), ii) deletion of T489 A490 Y491 T492 residues (sequence deleted 5′-ACAGCCTATACA-3′); and iii) insertion of residues SKCDCRGECFCD (sequence inserted: 5′-TCAAAATGTG ACTGCCGCGGAGAATGTTTCTGCGAC-3′) between T546 and P547 of the HI-loop. All modifications were done by homologous recombination in yeast [15].

The AdTATMMP was generated by inserting the TATMMP fragment from the plasmid pGEM-T fiber TATMMP end into the NcoI/MfeI sites of the pXK3.1 plasmid, which contains the wild type fiber, generating the pXK3.1–YTRGE–TATMMP. TATMMP fragment was obtained by cloning TATMMP sequence at the end of the fiber sequence by two consecutive Touch Down-PCR. First, TAT-like (TAT*) sequence was amplified from the plasmid pBSatYTRGE using the primers Fiber1Fw (5′-GCACCAAACACAAATCCC-3′) and FiberRv1 (5′GCGCCGCCTCTTCGTCGC TGTCTCCGCTTCTTGCTGCCATATTCTTGGGCAATGTATGA-3′). The PCR product, that contained the YTRGE mutations and TAT* sequence, was cloned into a T-vector following the manufacturer's instructions (pGEM®-T Easy Vector System I, Promega). Then, the MMP-cleavable linker-Blockage-3′ UTR sequence was amplified from the recently generated plasmid using the primers Fiber1Fw and FiberRv2 (5′-GCAATTGA AAAATAAACACGTTGAAACATAACACAAACGATTCTTTACTCCTCTTCCTCC TCGCCCTCTTCCTCGCCGCCCTTGTACAGGCCCTTGGCGCCGCCTCTTCGTC GCTGTCT-3′) and cloned into a T-vector generating the plasmid pGEM-T fiber TATMMP end. Next, homologous recombination of XbaI/KpnI digested TATMMP fragment with the linearized plasmid pVK50TL (adenoviral genome) was carried following a standard protocol. Viral particles were obtained and propagated in HEK293 cells.

The Ad^{RC}MMP is a replication-competent adenovirus, containing the wild-type Ad5 genome with the modified fiber TATMMP. It was generated by homologous recombination of XbaI/KpnI digested TATMMP fragment with the linearized plasmid pVK50 (adenoviral genome) following standard protocol. Viral particles were obtained in HEK293 cells and propagated in A549 cells.

All viruses were purified by standard cesium chloride banding and the physical particle concentration (vp/ml) was determined by optical density reading (OD_{260}). Viral titers for Adwt: $5{,}61 \times 10^{12}$ vp/ml and for Ad^{RC}MMP: $3{,}79 \times 10^{12}$ vp/ml. Correct fiber was verified by PCR and by PCR-product sequencing using Fiber1Fw, described above, and Fiber4Rv (5′-GTATAAGCTATGTGGTGGTGG-3′) primers.

2.3. Transduction efficiency assays

Cells were seeded in triplicate in 96-well plates. After 24 h, cells were infected with the corresponding adenovirus at 10^3 vp/cell or 10^4 vp/cell. Virus was removed 6 h later and cells were further cultured in a complete medium. Luciferase expression was measured three days later according to manufacturer's instructions (Luciferase Assay System; Promega). For experiments using pre-cleaved AdTATMMP, 24 h after cell seeding, AdTATMMP was incubated without or with different doses of MMP-9 (Calbiochem) in FBS-depleted medium, at 37 °C for 2 h, previous to cell infection.

2.4. In vitro cytotoxicity assays

A total of 3×10^3 cells (HT1080, PANC-1and RWP-1) or 10^3 cells (CAF-28) were seeded in triplicate in a 96-well plate and infected with either Adwt or Ad^{RC}MMP at different doses. Virus was removed 6 h later and cells were further cultured in a complete medium. Cell viability was measured 5 days later by an MTT colorimetric assay (Roche Molecular Biochemicals) (HT1080, PANC-1, and RWP-1) or by methylene blue staining (CAF-28).

2.5. Viral yield

PANC-1 and RWP-1 cells were seeded (4×10^4 cells per well in duplicate in 24-well plates) and infected with either Adwt or Ad^{RC}MMP at 50 vp/cell in the absence of FBS. Virus was removed 6 h later and cells were washed with PBS and cultured in FBS-complete medium. Total cell extracts were collected at 3 and 5 days post-infection and genomic DNA was extracted using UltraClean® BloodSpin® DNA Isolation Kit (Mo Bio Laboratories, Inc., Carlsbad, CA) according to the manufacturer's instructions. Viral genomes were determined by Real Time quantitative PCR (qPCR), performed on ViiA™ 7 System (Applied Biosystems, Life Technologies™) using SYBR Green mastermix (Roche) and hexon specific primers: AdHEXO1: GCCGCAGTGGTCTTAC ATGCACATC, AdHEXO2: CAGCACGCCGCGGATGTCAAAG. The adenovirus copy number was quantified with a standard curve, consisting of adenovirus DNA dilutions (10^2–10^7 copies) in a background of genomic DNA. Values were corrected by the number of cells per well, determined by qPCR of albumin intron 4. S-albumin: CTGTCATCTCTTGTGG GCTGT and aS-albumin: GGCTATCCAAACTCATGGGAG.

2.6. MMP expression analysis

Total RNA was obtained from pancreatic tissue with the RNeasy® Mini Kit (Qiagen) and reverse transcribed with Retroscript RT kit (Ambion, Life Technologies ™), using random decamers, in accordance with the manufacturer's protocols. Real Time quantitative PCR was performed on ViiA™ 7 System (Applied Biosystems, Life Technologies ™) using SYBR Green mastermix (Roche) and MMP-2 and GAPDH specific primers: MMP2-Fw: TGATGGCATCGCTCAGATCC, MMP2-Rv: CACAGC