Cancer Gene Therapy (2005), 1–9
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www.nature.com/cgt

Tumor cells as cellular vehicles to deliver gene therapies to metastatic tumors

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A long-pursued goal in cancer treatment is to deliver a therapy specifically to metastases. As a result of the disseminated nature of the metastatic disease, carrying the therapeutic agent to the sites of tumor growth represents a major step for success. We hypothesized that tumor cells injected intravenously (i.v.) into an animal with metastases would respond to many of the factors driving the metastatic process, and would target metastases. Using a model of spontaneous metastases, we report here that i.v. injected tumor cells localized on metastatic lesions. Based on this fact, we used genetically transduced tumor cells for tumor targeting of anticancer agents such as a suicide gene or an oncolytic virus, with evident antitumoral effect and negligible systemic toxicity. Therefore, autologous tumor cells may be used as cellular vehicles for systemic delivery of anticancer therapies to metastatic tumors.
Cancer Gene Therapy advance online publication, 14 January 2005; doi:10.1038/sj.cgt.7700801

***Keywords:* neoplasm metastasis; delivery system; oncolytic virus; NOD SCID mice**

Cancer is often diagnosed when the disease has already disseminated and most of the patient deaths are related to metastatic disease. Current treatment options for metastatic tumors lack efficacy and metastases targeting remains a major challenge for curing cancer. Novel cell- and gene-based therapies have been developed aimed to deliver an antitumor effect specifically to metastases. Some strategies rely on the immune system for selectivity, such as the stimulation of effector immune cells[1] or the use of monoclonal antibodies that recognize tumor antigens.[2] Others target angiogenic[3–5] or anaerobic signals[6,7] that arise at the metastases. Recently, oncotropic viruses have been used to selectively kill the tumor cells with the advantage that once targeting is achieved the propagation of the virus amplifies the therapy.[8,9] These promising new therapies have fallen behind expectations mainly because of their limited capacity for effectively targeting *in vivo*.[2,10]

The process of metastasis is a turning point in the progression of malignant solid tumors. Million tumor cells leave the primary tumor, reach the bloodstream and disseminate through the body.[11] Only a minority will eventually survive to become the origin of a new cancer nodule[12] at anatomical sites that are specific for the tumor type.[13] The dissemination of cancer cells from the primary tumor and their homing in specific organs involve several steps: invasion, detachment, circulation, cell adhesion, motility and invasion again. Cells must express a specific receptor molecule repertoire (cell adhesion molecules, chemokine receptors or integrin ligands among others) to complete this metastatic process.[14–17]

We hypothesized that cancer cells may be good candidates to target established metastases *in vivo* because they express the receptor and effector molecules involved in the metastatic process. Autologous intravenously (i.v.) injected tumor cells should respond to metastasis-related cues as the cells leaving the primary tumor did when the latter formed the metastases. In the present work, we demonstrated that i.v. injected tumor cells localized on established metastases and were able of carrying genetic-based antitumor agents such as suicide genes or oncolytic viruses to the metastases, delivering the therapy in a localized and toxic-tempered fashion.

Received May 31, 2004.
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Methods

Cell lines

The human breast cancer cell line MDA-MB-231 and the human prostate cancer cell line PC3 were maintained in DMEM supplemented with 10% fetal bovine serum

(FBS), glutamine and antibiotics (Life Technologies, Inc., Grand Island, NY).

Transduction of tumor cells with adenovectors or oncolytic adenoviruses

MDA-MB-231 or PC3 cells were transduced with different adenoviral vectors or oncolytic adenoviruses depending on the experiments. The adenovectors had either the *lac-Z* gene (AdLacZ), or the cytosine deaminase (CD) gene (AdCD[18]), under the control of the cytomegalovirus (CMV) promoter. The oncolytic adenovirus (AdwtFi-GFP) contains the wild-type E1 region and, after the fiber sequence (L5), the GFP coding sequence has been inserted with a splicing acceptor from the IIIa adenovirus gene. In this way, the adenovirus major late promoter regulates GFP, and viral replication can be followed by detection of green fluorescent cells.

The cells were transduced in DMEM, under conditions optimized so that 100% of the cells were infected: 1hour infection with 100 pfu/cell.[18]

Evaluation of bystander effect in vitro

Tumor cells were mixed with different proportions of AdCD transduced cells, and cultured in the presence of 400 μM 5-fluorocytosine (5-FC, Sigma Chemical Co., St Louis, MO). The cell viability was evaluated after 4 days by Trypan-blue (Sigma) exclusion of viable cells and crystal violet staining. In different experiments, tumor cells were mixed with different proportions of AdwtFiGFP-infected cells. The cell viability was evaluated after 6 days by Trypan-blue exclusion of viable cells and green fluorescence determination.

In vivo *studies*

We used 6–8-week-old NOD.CB17-Prkdcscid/J (NOD scid) mice, bred at the CIEMAT Laboratory Animals Facility (Registration Number 28079-21 A) from breeding pairs originally obtained from Jackson Lab (Bar Harbor, Maine). The mice were routinely screened for pathogens, in accordance with FELASA (Federation of European Laboratory Animal Science Associations) procedures. They were housed in microisolator individually ventilated cages, and allowed water and food *ad libitum*. All experimental procedures were carried out according to European and Spanish laws and regulations and internal biosafety and bioethics guidelines.

MDA-MB-231 cells were implanted in the mammary fat pad of female NOD/SCID mice while PC3 cells were implanted in the subcutaneous flank of male NOD/SCID mice. At indicated time points, the animals were euthanized by CO_2 inhalation. Samples from different organs were collected in 10% buffered formalin, Tissue-Tek (O.C.T. Compound, Zoeterwoude, The Netherlands) or immediately frozen.

Detection of tumor cells by RT-PCR

RNA was purified using the TRIzol reagent (Life Technologies). RT-PCR amplification of a human cytokeratin-19 sequence was carried out as published elsewhere.[18] β-gal+ cells were detected using the following *lac-Z* primers (sense: 5′CCGATCGCGTCACACTAC3′ and antisense: 5′CAGATGATCACACTCGGG 3′).

Histology and immunohistochemistry

Sections of paraffin-embedded organs were stained with hematoxilin–eosin, or with the CAM5.2 monoclonal antibody (anti-human pancytokeratin, Becton Dickinson, San Jose, CA) using a standard indirect avidin–biotin horseradish peroxidase method (Vectastain ABC kit, Vector Laboratories Inc., Burlingame, CA). Color was developed with diaminobenzidine (DAB, Vector Laboratories Inc.) and sections were counterstained with hematoxylin. Transduced tumor cells were stained with the anti-β-galactosidase Ab1 antibody (Oncogene, Cambridge, MA) and counterstained with eosin. GFP^+ cells were stained with a rabbit antiGFP IgG fraction (Molecular Probes, Eugene, OR) and counterstained with hematoxylin.

Cryosections were stained with X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactoside, United States Biochemical Corp., Cleveland, OH) and then with the CAM 5.2 antibody, and counterstained with eosin.

Evaluation of lung area, lung metastases and localization of the i.v. injected tumor cells

Images of the lung cryosections stained with the CAM 5.2 antibody ($n=10$, two animals) were digitalized and analyzed with the IMAGE-PRO Plus 4.5 software (Media Cybernetics Inc., Carlsbad, CA). Different colors were assigned to healthy and to metastatic tissues, and the total lung and metastatic areas were calculated in square pixels for each color, by an independent researcher (Dr José M Martínez, departamento de Ciencias de Materiales, Universidad Politécnica de Madrid). The associated error was estimated as less than 4% in triplicate measurements.

The number of β-gal+ cells and its localization in the lungs were directly scored under a light microscope on lung cryosections stained with X-gal and the CAM 5.2 antibody ($n=10$, two animals). Colocalization was defined as the presence of a β-gal+ cell directly in contact with a cytokeratin-positive nodule. All other situations were not considered as colocalization.

Evaluation of bystander effect in vivo

(a) *Cells transduced with the AdCD vector*. Metastatic tumors were generated as explained before. The primary tumors were excised and a group of mice received five doses of 3×10^5 transduced cancer cells, two doses per week. An osmotic pump (Alza Corporation, Palo Alto, CA) delivering 5-FC at a rate of 0.25 μL/hour 5-FC (10 μg/mL) was subcutaneously implanted in the animals. At 10 days after the last i.v. injection, the mice were killed, the diameters of the remaining primary tumors were scored and the organs were recovered for histology and quantification studies. Metastatic burden was evaluated on DNA samples from the organs by dot blot hybridiza-

tion with a human specific probe (a kind gift of Dr John Dick).

(b) *Cells infected with the AdwtFiGFP virus*. Metastatic tumors were generated as explained before. The primary tumors were excised and a group of mice received five doses of 3×10^5 transduced cancer cells, two doses per week. At 10 days after the last i.v. injection, the mice were killed, the fresh organs were analyzed under a fluorescent light and the lungs were weighed and recovered for immunohistochemical analysis.

Statistical analysis

Statistical analysis was performed using the Stata software program (Stata Press, College Station, TX). Results are expressed as mean $\pm$ standard error (SE). Results were considered significant if the *P* value was equal to or less than .05. We used the two-sample Wilcoxon rank-sum test (Mann–Whitney two-sample statistic) for comparisons.

Results

Tumor cells colocalized on pre-existing metastases

We generated metastatic cancer in mice by implanting MDA-MB-231 human breast cancer cells in the mammary fat of female NOD/SCID mice. In this model, human breast cancer cells formed a primary tumor in the mammary fat, and metastasized preferentially to the local lymph nodes, lungs, kidneys, liver and other organs (Fig S1). In a different experiment, we determined whether i.v. injected tumor cells would localize on pre-existing metastases. In mice with metastatic disease, we injected i.v. a single dose of MDA-MB-231 cells transduced with an adenoviral vector carrying the *lac-Z* transgene. Transduction conditions were optimized so that all the injected cells expressed the transgene.[18] At 24 hours after i.v. injection, we determined the localization of the transduced cancer cells. Immunohistochemical analysis showed β-gal+ cells in micrometastatic nodules of the lungs and kidneys (Fig 1). Microscopic examination (serial sections of 5 μm) showed that 46% of the β-gal+ cells in the lungs colocalized with micrometastases. A total of 19% of the total lung area was tumoral and an average of 20% of the lung metastases had β-gal+ cells after a single i.v. injection of 3×10^5 transduced tumor cells. We did not detect β-galactosidase activity (X-gal staining) in the preparations we studied from the kidney (10 sections), liver (10 sections) and cerebrum (10 sections). Using a different detection technique, we confirmed the presence of β-gal+ cells in the primary tumor and in organs with metastases by RT-PCR of the *lac-Z* transgene (Fig S1). These experiments demonstrated that i.v. injected tumor cells targeted metastatic lesions. In addition, injected tumor cells carried a heterologous molecule to the metastases. Preliminary experiments using the same approach with PC3 (human prostate) cancer cells in male NOD/SCID mice confirmed that i.v. infused cancer cells localized on established metastases.

Ex vivo *manipulated tumor cells exerted a bystander toxic effect* in vitro *and* in vivo *on the metastases*

In the light of these findings, we used the tumor cells for cancer treatment. MDA-MB-231 or PC3 cancer cells were transduced with an adenoviral vector carrying the *Escherichia coli* enzyme cytosine deaminase (CD) (AdCD). The cells expressing the CD transform the prodrug 5-FC into the anticancer drug 5-fluoruracil (5-FU), which eliminates the transduced cells and the cells around them (bystander effect). Transduced cells were mixed with different proportions of untransduced cells and cultured in the presence of 5-FC. The viability of the mixture decreased when 5% of cells in the starting cultures were transduced (Fig S2). We next studied whether the *ex vivo* manipulated tumor cells have therapeutic effect *in vivo*. We generated metastatic cancer in mice (as explained) and, when the primary tumor reached a major axis of 10 mm, we surgically eliminated as much tumor as possible before delivering the treatment. For treatment, we implanted an osmotic pump delivering 0.25 μL/hour 5-FC (10 μg/mL) for 4 weeks in a first group of mice, and i.v. injected them with five doses of MDA-MB-231 cells transduced with the AdCD vector (two doses per week, 3×10^5 cells per dose). At 10 days after the last i.v. injection, the mice were killed and analyzed. The macroscopic examination showed that the treated mice had a significant smaller primary tumor volume than the untreated group (received only surgery). Large infiltrated lymph nodes, local and distant to the primary tumor, could be seen in the animals of the control group but were absent in the treated mice. Histological examination of the lungs showed abundant large cancer nodules in the untreated mice while the treated mice had no visible nodules (Fig 2a). Moreover, we quantified the amount of cancer in the organs prone to metastasis and found that the treatment had diminished the metastatic burden in the lungs and in kidneys when compared with the control group (Fig 2b). We did not detect tumor cells in the control mice that received only transduced tumor cells i.v. and the implantation of the 5-FC osmotic pump (not shown).

Ex vivo *manipulated tumor cells carried an oncolytic adenovirus to the metastases*

Aiming to validate this experimental approach with a different antitumor agent, the i.v. injected cancer cells were infected with oncolytic adenoviruses. We used a replication-competent adenovirus, AdwtFiGFP. Following the sequence encoding the fiber (L5), this vector contains an adenovirus IIIa splicing acceptor and the GFP coding sequence (such an L6 unit). Therefore, GFP is expressed as another late protein upon replication. We expect this adenovirus to replicate in tumor cells, lyse them and spread to the surrounding tumor cells. Viral replication can be followed by detection of green fluorescent cells. We used it as a proof of concept that tumor cells may transport oncolytic viruses to the metastases. In our model, the AdwtFiGFP cannot replicate in murine cells but it does in the human tumor

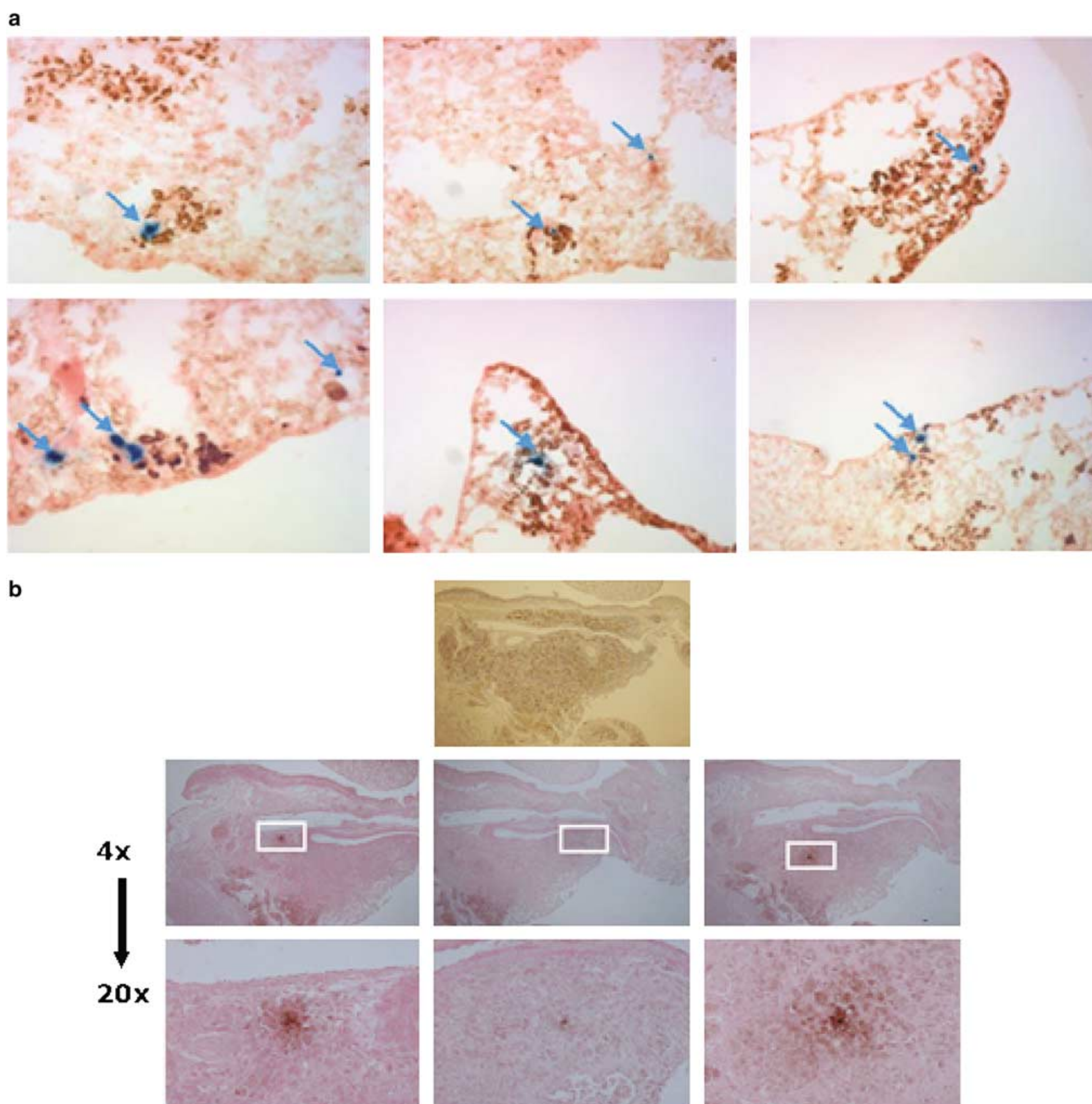

Figure 1 I.V. injected tumor cells localized on established metastases. (**a**) Lung cryosections stained with X-gal and the CAM5.2 antibody (anti-human pancytokeratin) showed β-gal+ cells (i.v. injected) in or by established metastases. (**b**) Above: a low-power view showing the hematoxilyn–eosin and CAM 5.2 antibody stain for the tumor nodule. Below: three different paraffin-embedded sections of the metastasis stained with an anti-β-galactosidase antibody. Positive cells were detected at different levels within the tumor nodule.

cells. In preliminary *in vitro* experiments to assess the bystander effects of this strategy, MDA-MB-231 and PC3-infected cells were mixed with different proportions of uninfected cells. The infection kinetic was followed by periodical analyses under a fluorescence microscope. The viability of the mixtures was determined after 6 days and decreased when as low as 5% of the infected cells were present in the culture (not shown). Longer culture times ended up with a complete mortality of the mixtures. In a set of mice with metastatic cancer, after surgical elimination of their mammary fat pad tumors (as above), we injected i.v. cells carrying the AdwtFiGFP adenovirus. The mice received five doses of infected cells (two doses per week, 3×10^5 cells per dose) and 10 days after the last dose were killed and their fresh organs examined under fluorescent light (Fig 3a). We found nodules expressing the GFP in the lung and liver of three out of seven mice, and confirmed that the images corresponded to tumor

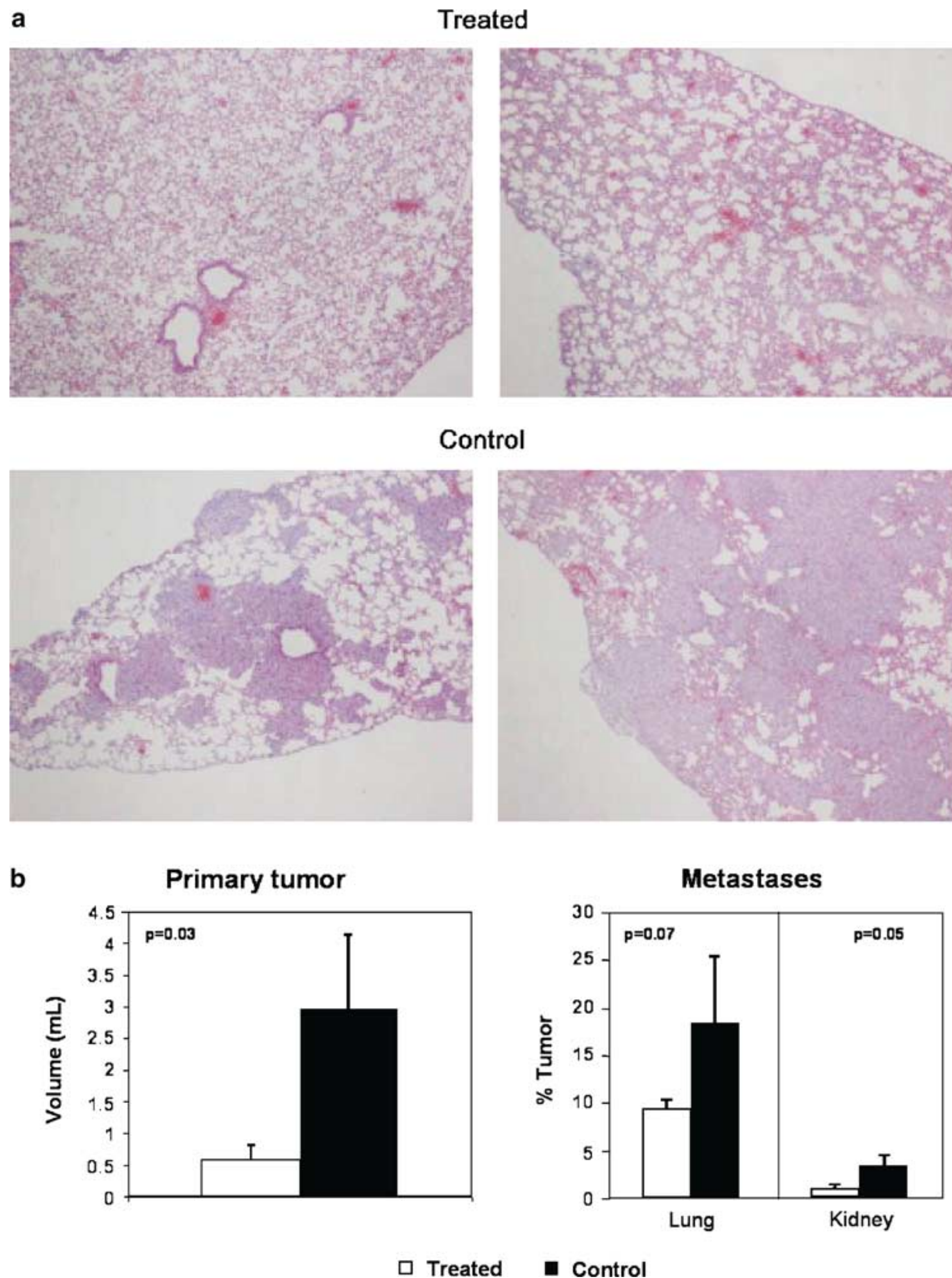

Figure 2 *Ex vivo* manipulated tumor cells exerted a bystander toxic effect on the metastases. (**a**) Hematoxilin–eosin colored sections of paraffin-embedded lungs showed the presence of abundant metastases in the control mice but not in the treated ones. (**b**) Tumor volumes were estimated by the formula $V = (\pi/6)a^2b$, where "*a*" was the short axis and "*b*" the long axis (left). Metastatic burden was quantitated as percentage of human DNA by dot blot with a human-specific probe on DNA samples from the lungs and kidneys (right) ($n = 5$ mice per group).

nodules by RT-PCR of the human cytokeratin-19 gene. Green nodules were not seen in control mice (received surgical treatment) or mice injected only with the infected cells (the AdwtFiGFP does not replicate in murine cells), indicating that the green nodules corresponded to metastases where AdwtFiGFP was actively replicating. Immunohistochemical staining with an anti-GFP antibody revealed the presence of GFP-positive tumor cells in the middle of GFP-negative micrometastatic nodules (Fig S3). These results showed that i.v. injected tumor cells initiated an oncolytic infection and that the lytic process propagated to the surrounding tumor mass. This oncolytic infective process was active long after the last i.v. injected cells had died (*in vitro* cell lysis happened within 96 hours), and resulted in a significant reduction in the lung metastases of the treated mice compared to the control mice (Fig 3b). Our results indicate that effective targeting could be achieved with this delivery system and virus replication in metastases was revealed by the presence of GFP.

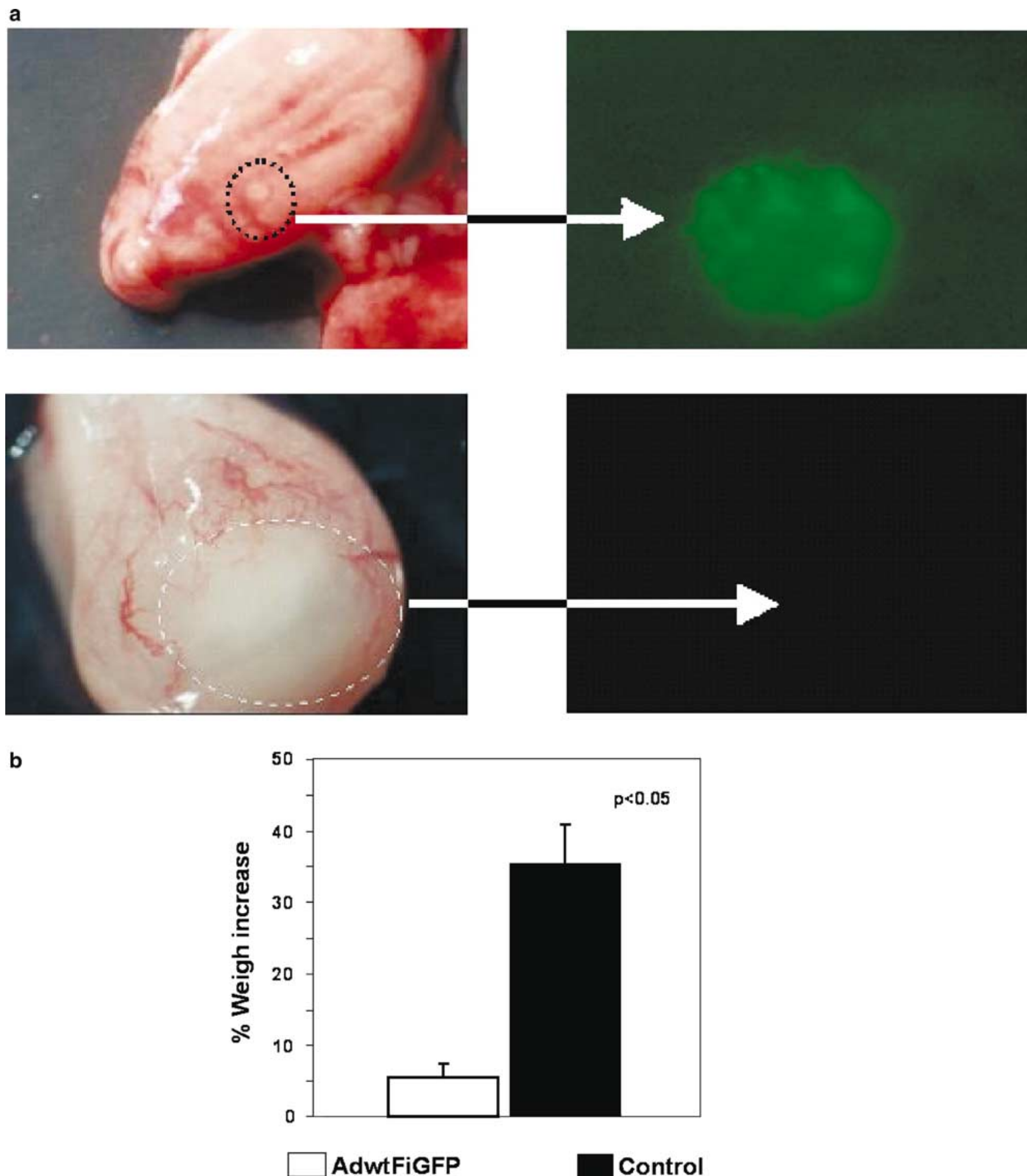

Figure 3 *Ex vivo* manipulated tumor cells carried an oncolytic adenovirus to the metastases. (**a**) Upper: a cancer nodule in the lung surface of a treated mouse is shown (left), and the same nodule is under fluorescent light (right). Bottom: a cancer nodule in the lung surface of a control mouse (left). The same nodule under fluorescent light (right). (**b**) Lungs were weighed after killing. Weight increase was calculated in comparison to healthy mice ($n=7$ mice per group).

The therapeutical effect of i.v. injected tumor cells has no detectable systemic toxicities

Microscopic examination of histological sections of the organs from the treated mice did not show evidence that the treatment had been toxic to the animals. We further evaluated the toxicity associated to the treatment with i.v. injected tumor cells by analyzing the renal and hepatic functions. For this experiment, we did not generate any

Table 1 Biochemical evaluation of liver and kidney functions after treatment

	ALT	γGT	Bilirubin	Protein	Creatinine	Urea
(a) acute phase (10 days after last i.v. injection) and (b) chronic phase (50 days after last i.v. injection)						
(a) Untreated	18.7±6	4.7±3	0.44±0.1	4.74±0.2	0.31±0.05	42.7±1.1
AdCD treated	14±7	5.3±4	0.34±0.2	4.12±0.6	0.3±0.02	38.3±2.9
(b) Untreated	13.4±1.3	4.4±1.9	0.5±0.2	5±0.4	0.3±0.02	49±5.3
AdCD treated	9.5±6.6	7.2±1.9	0.4±0.2	4.7±0.3	0.4±0.05	39.3±4.2

ALT: alanine transaminase (U/L); γGT: gamma glutamil transferase (U/L); bilirubin (mg/dL); protein: (g/mL); creatinine: (mg/dL); urea: (mg/dL).

metastatic cancer in the mice before the i.v. injections, but mice received the same treatments as above. Biochemical parameters of both renal (urea and creatinine) and hepatic (transaminase enzymes, total proteins, bilirrubin and γGT enzyme) functions were not affected by the treatment (Table 1).

Discussion

We show here that i.v. injected tumor cells home in established metastases. Our preliminary quantification indicated that half of the β-gal+ cells in the lungs localized in established metastases. Given that only 19% of the total lung area were occupied by metastases, this number suggests that tumor targeting was not a random process. It has already been reported that intraperitoneally injected ovarian carcinoma cells preferentially localized at sites of locoregional metastatic ovarian cancer,[19] showing that injected cancer cells interacted with established tumors. In addition to the lungs, we found β-gal+ cells in different sections of a kidney metastasis (Fig 1). Thus, localization cannot be solely explained by anatomical factors (route of injection and first pass effect). We foresee three steps at which targeting may take place. First, i.v. injected tumor cells may respond to the chemokines involved in the preferential homing of metastatic cells in organs.[16] Second, i.v. injected tumor cells may attach to the vascular endothelium similarly to intravascular micrometastases.[20] Last, i.v. injected tumor cells should be able to participate in the crosstalk between the cancer cells and factors from the host tissues that governs the extravasation of the metastases.[21,22] Supporting this, *in vitro* experiments have shown that noninvasive cancer cells can progress through three-dimensional matrices following the track of invasive tumor cells, which suggests that tumor cell migration and invasion may be modulated by signals generated by other tumor cells.[23] Infiltrating central nervous system tumors give rise to signals, which can be read and followed by specific neural cell types,[24] underscoring the role of tumor–host signals for recruiting other cells into the metastases environment. It is reasonable to assume that transducing tumor cells with genes implicated in the metastatic process would likely enhance the targeting efficacy.

Different cell types have been used as vector carriers for systemic cancer gene therapies.[25] These cellular vehicles act like shuttles for delivering vectors to the sites of tumor growth much more efficiently than the vectors *per se* regularly accomplish. Injection of oncolytic adenoviruses to the circulation is followed by viral sequestration in the liver.[8] The effective targeting of metastases when injecting monoclonal antibodies is just 0.001–0.01% of the total injected dose.[2] Therefore, there is a growing need of vehicles that enhance the efficacy of new oncolytic viruses or monoclonal antibodies.[2,10] Allogeneic tumor cells have been tested as vehicles in locoregional tumors[19] but not as systemic carriers. We present here two examples of how *ex vivo* manipulated autologous tumor cells may deliver a localized anticancer effect *in vivo* after systemic delivery. Using autologous rather than allogeneic tumor cells will prevent an immune rejection against the anticancer cellular vehicles. In the case of AdCD-transduced cells, locally produced 5-FU would be responsible for the killing of the metastases, although a systemic effect of 5-FU may also participate. The highest levels of the drug would be reached at the site where it is produced, that is, the injected cells. High levels of 5-FU from transduced tumor cell should reach the metastases immediately after being produced. The CD/5-FC system does not require intercellular gap junctions, often lost in tumor cells,[26] for bystander effect. This gives advantage over other suicide gene therapy systems, such as the HSV-TK. Antitumor immunity has been associated with the destruction of tumor cells with the CD/5-FC gene therapy[27] and may enhance the therapeutic benefits. As we used immunodeficient mice we could not evaluate this possibility. We used the AdwtFiGFP adenovirus as a model of oncolytic virus. Most of the advances on oncolytic adenoviruses have been related to replication selectivity through mutations or promoter insertions but a major hurdle remains with regard to tumor targeting.[10] Our studies focus on the delivery and detection of viral replication in the targeted metastases and we used this kind of virus, a replicating vector with no such mutations or promoter insertions, as a proof of concept. Tumor cells present a major advantage as vehicles for oncolytic viruses since they are the primary cell types where the viruses replicate. Candidate anticancer agents that would fit in our strategy are suicide genes, antiangiogenic genes, oncolytic viruses, monoclonal antibodies or drugs.

Safety is likely the most important concern when considering new therapies. Our preliminary results about

systemic toxicities are encouraging, however, the potential danger related to the infusion of live tumor cells back into the patient needs to be addressed in further research. It is well known that metastasis is a very inefficient process,[12,22] even so, injected tumor cells may cause an unwanted bystander effect on healthy cells, since targeting is not totally specific. Risks can be greatly minimized before infusionly optimizing the transduction efficiency up to 100% as shown here, selecting the transduced cells and inactivating the cells (lethal irradiation, chemicals). Loading cancer cells with selective antitumor agents will also enhance safety, that is, oncolytic viruses provide a self-destructive mechanism in this strategy.

We consider our strategy as a treatment for the metastatic disease rather than for the primary tumor, even though we detected that i.v. injected tumor cells localized in the primary tumor. Surgery of the primary tumor is a standard first-line treatment for patients with cancer, although it stimulates neoangiogenesis and growth of metastases.[28] In this sense, our model resembles the clinical situation of many patients with solid tumors. The strategy shown is rationally different from the actual anticancer therapies (surgery, chemoradiotherapies) and they could be employed synergistically. We anticipate that tumor cells may be loaded with different anticancer agents, as unique or combined therapeutic elements, and deliver its beneficial effect locally with less toxicity. In summary, we show here that autologous tumor cells may be used as a flexible tool for the treatment of the metastatic disease. Further research is needed for addressing crucial points before its use in patients. We have designed experiments to rule out the ability of lethally irradiated MDA-MB-231 cells to target metastatic lesions. Chasing experiments with a different tumor cell line will help in clarifying the target specificity. In addition, the experiments will be performed in immunocompetent models with murine tumors in order to determine the role of the immune system.

Acknowledgments

We thank Isabel de los Santos, Pilar Hernández and Sergio García for technical assistance, and Dr José M. Martínez for his help with analyzing image files. This work was supported in part by Fundación Leucemia Linfoma (MR) and Fundación Oncohematología Infantil (JGC, LM and MR).

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Supplementary information accompanies the paper on Cancer Gene Therapy website (http://www.nature.com/cgt).