Macrophage Delivery of an Oncolytic Virus Abolishes Tumor Regrowth and Metastasis after Chemotherapy or Irradiation

Munitta Muthana1,2, Samuel Rodrigues2, Yung-Yi Chen1, Abigail Welford1, Russell Hughes1, Simon Tazzyman1, Magnus Essand3, Fiona Morrow2, and Claire E. Lewis1

Abstract
Frontline anticancer therapies such as chemotherapy and irradiation often slow tumor growth, but tumor regrowth and spread to distant sites usually occurs after the conclusion of treatment. We recently showed that macrophages could be used to deliver large quantities of a hypoxia-regulated, prostate-specific oncolytic virus (OV) to prostate tumors. In the current study, we show that administration of such OV-armed macrophages 48 hours after chemotherapy (docetaxel) or tumor irradiation abolished the posttreatment regrowth of primary prostate tumors in mice and their spread to the lungs for up to 27 or 40 days, respectively. It also significantly increased the lifespan of tumor-bearing mice compared with those given docetaxel or irradiation alone. These new findings suggest that such a novel, macrophage-based virotherapy could be used to markedly increase the efficacy of chemotherapy and irradiation in patients with prostate cancer. Cancer Res; 73(2): 490–5. ©2012 AACR.

Introduction
Solid human and murine tumors often respond well initially to conventional, frontline therapies such as chemotherapy and radiotherapy, leading to the cessation of tumor growth and even tumor shrinkage. However, a major clinical problem is the subsequent regrowth of such tumors—both at the site of the primary tumor and/or distant sites. This relapse results in patients receiving multiple rounds of the same or different therapies.

Monocytes are continually recruited into tumors where they differentiate into tumor-associated macrophages (TAM) and accumulate in poorly vascularized, hypoxic areas (1, 2). We showed previously that macrophages can be used to deliver a hypoxia-regulated, prostate-specific oncolytic virus (OV) to such sites in prostate tumors (3). To do this, macrophages were cotransduced with a hypoxia-regulated E1A/B construct and an E1A-dependent oncolytic adenovirus. The proliferation of the virus was also restricted to prostate tumor cells using prostate-specific promoter elements from the TARP, PSA, and PSMA genes. When such cotransduced cells were injected into tumor-bearing mice, they protected the virus from neutralizing antibodies in the circulation and delivered it to tumors. Once inside hypoxic tumor areas, E1A/B proteins were expressed by the cotransduced macrophage, activating replication of the adenovirus. This was then released and infected neighboring tumor cells in both hypoxic and well-oxygenated areas of tumors, replicated further and lysed each new host cell. This then resulted in the marked inhibition of both primary tumor growth and the formation of pulmonary metastases (3).

Both chemotherapy and tumor irradiation are now known to cause not only the formation of large areas of tumor hypoxia and necrosis (4, 5) but also a marked increase in macrophage recruitment by tumors (6–9). The aim of the current study was to see if this therapy-induced macrophage recruitment could be exploited to deliver a second, potent therapeutic insult to tumors after therapy—large quantities of an oncolytic virus—and, in doing so, markedly increase the efficacy of such standard therapies. Injection of cotransduced macrophages were administered 48 hours after chemotherapy (docetaxel) and tumor irradiation and found to halt both the regrowth and metastatic spread of human prostate tumor xenografts after these therapies.

Materials and Methods
Mouse procedures and human monocyte isolation were conducted in accordance with the University of Sheffield Ethics Committee and UK Home Office Regulations.

Isolation of human monocytes and generation of monocyte-derived macrophages
Macrophages were prepared from mononuclear cells isolated from buffy coats (Blood Transfusion Service; ref. 3).
Cotransduction of primary monocyte-derived macrophages

To prevent undesirable viral recombination events, the HRE-regulated E1A/B gene constructs were transferred into macrophages by plasmid transfection rather than coinfection with a second viral vector. For cotransduction, monocyte-derived macrophages (MDM; 2 x 10^6) that had been cultured for 3 days were infected with adenovirus with a multiplicity of infection (MOI) of 100 plaque-forming unit (PFU)/cell and incubated overnight and then transfected with 5 μg pcDNA3.1(+)–HRE-E1A/B (HRE-E1A/B) construct using the Amaglo Macrophage Nucleofection Kit (Amaxa Biosystems). Optimal transduction of MDMs was determined using a reporter adenovirus (AdCMV-GFP). This was achieved with an MOI of 100 PFU/cell as measured by flow cytometry for expression of GFP (3).

Mice

Male CD1 athymic mice were used in these studies (Charles Rivers). LNCaP-LUC cells were obtained from Professor Magnus Essand (Uppsala, Sweden; ref. 10). The cells were cultured in RPMI-1640 supplemented with 10% FBS in a humidified, 5% CO₂ atmosphere at 37°C. The mouse cells are routinely tested for authenticity by microsatellite genotyping at the ECACC and mycoplasma testing (GENEFLOW).

Orthotopic prostate xenograft model. One million LNCaP-LUC cells were mixed 1:1 in Matrigel and injected into the dorsolateral prostate. Tumor take was monitored by bioluminescence imaging using the IVIS Lumina II imaging system (Caliper Life Sciences). This detects live luciferase-bioluminescence imaging using the IVIS Lumina II imaging system. Mice were transferred to the dark box and iso

Irradiation studies

Male CD1 athymic mice (Harlan laboratories) were injected subcutaneously with 2 x 10^6 LNCaP-LUC cells mixed 1:1 with Matrigel (BD Biosciences) into the hind flank region. Male CD1 athymic mice were injected subcutaneously with 2 x 10^6 LNCaP-LUC cells mixed 1:1 with Matrigel (BD Biosciences) into the hind flank region. This was to allow ease of access for tumor irradiation. When tumors reached 4 mm in diameter, mice received a single dose of 20 Gy radiation therapy. Restraining chambers designed to expose only the flank area of mice were used to allow highly localized irradiation of tumors. The cumulative growth of tumors was evaluated and compliance with UK Home Office Regulations, were removed by day 14 due to their large size. After 48 hours, this was followed by tail vein injection with 100 μL PBS containing 3 million cotransduced macrophages (Ad[I/PPT-E1A]) or reporter AdCMV-GFP at MOI 100 and HRE-E1A/B, 5 x 10^10 Ad[I/PPT-E1A] only or PBS alone as described above. Tumor size was determined using calipers. Again, animals were sacrificed once tumors reached the maximum permitted volume and tumors/organs were excised and processed as above.

Histology

Five-micrometer paraffin wax sections from tumors and tissue were cut, dewaxed, rehydrated, and stained with hematoxylin and eosin to enable areas of tumor necrosis to be readily visualized using morphologic criteria—reduced cellular density, pale cytoplasm, and pyknotic nuclei or completely disrupted cells, with or without red blood cell infiltration. Hypoxia-bound pimonidazole (PIMO) was detected in tumor sections using Hypoxyprobe-1MAb1, a monoclonal antibody IgG1 (Millipore). PIMO labeling was then quantified across whole tumor sections using a random point scoring system based on that described by Smith and colleagues (11). Sections were also incubated with specific antibodies for target antigens; CD31 (1:100), F4/80 (1:80; AbD Serotec), human C668 (Dako) at 1:100 and E1A at 1:50 (Millipore). A biotinylated secondary antibody system was used in conjunction with a streptavidin-conjugated horse-radish peroxidase. Peroxidase activity was localized with diaminobenzidine (Vectastain Elite ABC kit, Vector Labs). Metastatic burden was assessed by serial sectioning of formalin-fixed, paraffin-embedded lung tissue whereby the entire lung was sectioned and the number of metastatic foci (>5 cells) was determined on 5 sections taken every 100 μm. Human LNCaP-LUC cells within the lungs were identified by staining with anti-human EpCAM using the immunohistochemical procedure described above. All immune
localization experiments were repeated on multiple tissue sections and included isotype-matched controls for determination of background staining.

For details of plasmid construction, the oncolytic adenovirus used, generation of MDMs, cotransduction of primary MDMs, assessment of tumor necrosis/hypoxia, tumor metastasis and immunolabeling/analysis of CD31, F4/80, CD68, E1A & Ep-CAM in tumor sections, please see the work of Muthana and colleagues (3) and Supplementary File.

Statistical analysis

In most cases, multiple comparisons of groups was conducted by ANOVA followed by the Tukey–Kramer honest significance difference test (GraphPad Software Inc.). All data represent mean values ± SEM and P < 0.05 was considered to be significant. Data in Figs. 1–3 are from a single experiment but essentially similar results were achieved when this was repeated.

Results and Discussion

Three intravenous injections of docetaxel significantly ($P < 0.03$) delayed the growth of orthotopic prostate (LNCaP-LUC) tumors until day 14 (i.e., 10 days after the last docetaxel injection; Fig. 1A and Supplementary Fig. S1A) and improved mouse survival (Fig. 1B). However, tumors then regrew over the next 7 days (i.e., between days 14 and 21)—with increased tumor hypoxia and necrosis evident by day 35 (compared with tumors from the “vehicle” group removed by day 14 due to their large size; Supplementary Fig. S1B and S1D), as was a marked tumor infiltration by murine TAMs by day 2, which was still present at day 35 (Fig. 1C). This concurred with our previous pilot studies (data not shown) and confirmed that tumor infiltration by our infused OV-bearing macrophages was likely to take place if they were injected systemically 2 days following the last of the docetaxel injections. So, this time point was selected for a single

![Figure 1](link-to-image)

Figure 1. Macrophage delivery of an oncolytic virus (Ad[1/PPT-E1A]) abolishes the regrowth of human prostate (LUC-LNCaP) tumors after treatment with the cytotoxic agent, docetaxel (DOX). Tumor-bearing mice were administered with 3 doses of DOX (10 mg/kg) by i.p. injection on days 0, 2, 4 and then injected i.v. on day 6 with a single dose of either the OV alone or cotransduced MDM. A, tumor luminosity showed that DOX alone prevented tumor growth up to 14 days, but they then rapidly regrew. OV alone delayed this regrowth for up to 7 days, but delivery of the OV via cotransduced MDMs completely abolished it for 35 days. Circles, time points when tumors were taken for analysis. B, mouse survival (NB, the final data points on each line were when mice were culled). C, quantitative analysis of 6 high-power fields (HPF; ×20 magnification) per tissue section from 5 mice per group revealed murine F4/80+ TAMs increased significantly within 2 days of DOX and after injection with DOX + OV. D, OV infection (viral E1A protein staining; red, see arrows) occurred in tumors after DOX + OV alone but was higher in the DOX + cotransduced macrophage group. Representative data shown for 1 of 2 replicate experiments where n = 5 mice/group. Data are means ± SEM. Statistical significance differences **, $P < 0.05$; ***, $P < 0.01$ compared with DOX alone group; **, $P < 0.01$ compared with DOX + OV group. Bar, 200 μm.
injection of OV-carrying macrophages (and for the purpose of comparison, "free" OV was administered to a separate group). OV alone significantly ($P < 0.01$) delayed tumor regrowth after docetaxel by 7 days (only occurring between days 21 and 28), whereas macrophage delivery of OV completely ($P < 0.0002$) abolished tumor regrowth for up to day 35 and extended the survival of tumor-bearing mice (Fig. 1A and B). Both OV treatments significantly ($P < 0.001$) reduced microvessel density in docetaxel-treated tumors (sampled at day 28 for the docetaxel + free OV group and day 35 for the docetaxel + cotransduced macrophages) compared with docetaxel alone group (at day 28) but failed to affect tumor hypoxia (Supplementary Fig. S1C and S1D). Human CD68$^+$ macrophages were present in tumors in mice injected with either GFP-expressing or OV-bearing macrophages (Supplementary Fig. S1E), and the latter resulted in significantly ($P < 0.001$) more OV detection throughout tumors after docetaxel than in the tumors of mice injected with free OV alone + docetaxel (Fig. 1D).

A single dose of 20 Gy radiotherapy (RT) was also seen to significantly reduced the growth of LNCaP:LUC tumors for 21 days but they then started to regrow and had to be removed at day 35 (i.e., when they reached the tumor size of those in the PBS alone group at day 14). A similar pattern of tumor regrowth occurred in mice receiving RT followed 2 days later by "control" macrophages (i.e., transfected to express the reporter gene, GFP; Fig. 2A and Supplementary Fig. S2A). Thirty-five days after RT, there was a small but insignificant drop in tumor microvessel density compared with that in tumors from the PBS alone group, along with a significant ($P < 0.01$) increase in tumor necrosis and hypoxia in the RT alone group (Supplementary Fig. S2B–S2D). Although these effects in the RT-
treated tumors are consistent with those reported for RT in other mouse tumor models (4, 9), it should be noted that they may reflect the effects of tumor regrowth after RT treatment on tumors, rather than RT per se.

Two days after RT, a marked tumor infiltration by murine F4/80+ (i.e., host) macrophages occurred and the number of these cells was still elevated at day 35 (Fig. 2C). Again, this concurred with our previous pilot studies (data not shown) and suggested that tumor infiltration by our OV-bearing macrophages would be highly likely to occur if these cells were injected systemically within 2 days of RT. So, as in the docetaxel study, this time point was selected for the single injection of OV-carrying macrophages (or "free" OV). Whereas a single, systemic injection of the latter delayed tumor regrowth after RT by 7 days (so mice could be sacrificed at day 42 rather than 35), OV delivery via cotransduced macrophages significantly extended this, with no regrowth evident by 42 days after RT (i.e., the end of the experiment; Fig. 2A and Supplementary Fig. S2A). This correlated with improved survival rates in the latter group, compared with those receiving RT with free OV (Fig. 2B). There were also significantly (P<0.001) fewer CD31+ blood vessels and more necrosis in tumors receiving macrophage-delivered OV than OV alone (Supplementary Fig. S2B–S2D). Following RT, human CD68+ macrophages were present in tumors receiving macrophage-delivered GFP and OV (Fig. 2C and Supplementary Fig. S2E), leading to widespread expression of OV in the latter group (Fig. 2D).

We next determined how these therapies influenced the development of pulmonary metastases. Few metastases were detected in mice injected with PBS alone (no docetaxel) because, as mentioned previously, primary tumors in this group had to be removed by day 14 (due to their size). Therefore, it was not valid to compare metastases in this control group with the 4 experimental groups. Metastases form in the lungs by day 21 in the LNCaP model used in these studies (Fig. 3A). The formation of lung metastases after docetaxel was abolished when OV-bearing macrophages were injected 2 days after the final docetaxel was delivered (a phenomenon not seen when mice were injected with free OV or macrophages bearing a control, GFP-expressing adenovirus; Fig. 3A and B). OV was detected in some areas of lung in mice injected with OV-bearing macrophages after docetaxel but not docetaxel + free OV (Supplementary Fig. S4A). Similarly, pulmonary metastases were significantly

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(P < 0.0012) higher in mice receiving RT + free OV than in RT + cotransduced macrophages (a valid comparison as both groups were sacrificed on day 42; Fig. 3C and D). OV (E1A staining) was detected in some small areas of the lungs of mice injected with OV-bearing macrophages after RT but not in mice injected with free OV after RT (Supplementary Fig. S4B). In addition to targeting the primary tumor, we believe that our macrophage-based therapy homed to pulmonary metastases and prevented their development.

The use of athymic (nude) mice in the above xenograft tumor model meant we could not assess the immune response of host mice to the cotransduced, human macrophages, immunocompetent mice could not be used because murine cells, including macrophages, do not support adenoviral replication. For this reason, we decided to use a well-characterized human prostate xenograft model rather than a transgenic mouse tumor model (orthotopic in the case of the docetaxel study). It should be noted that the majority of preclinical studies of anticancer gene therapies have used similar human tumor xenograft models (12, 13).

In sum, the current study shows that it is possible to exploit the increased macrophage infiltration in tumors that occurs after chemotherapy or irradiation to deliver a macrophage-based OV therapy. This profoundly suppressed the regrowth and metastatic spread of human prostate tumor xenografts after such frontline therapies. Further studies are now warranted to see if such a combined therapeutic approach will be equally effective in patients with prostate cancer.

**Disclosure of Potential Conflicts of Interest**
No potential conflicts of interest were disclosed.

**Authors’ Contributions**

Conception and design: M. Muthana, Y.-Y. Chen, C.E. Lewis

Development of methodology: M. Muthana, Y.-Y. Chen, F. Morrow, C.E. Lewis

Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): M. Muthana, S. Rodrigues, Y.-Y. Chen, R. Hughes, S. Tazzyman

Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): M. Muthana, Y.-Y. Chen, A. Welford

Writing, review, and/or revision of the manuscript: M. Muthana, Y.-Y. Chen, C.E. Lewis

Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): M. Essand, F. Morrow

Study supervision: M. Muthana, Y.-Y. Chen, C.E. Lewis

Developed and produced the oncolytic virus used in the study and the luciferase-expressing target cell line: M. Essand

Conducted the staining and support practical work, analysis of images, and preparation of macrophages and virus: F. Morrow

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