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

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Abstract

Frontline anti-cancer therapies like chemotherapy and irradiation often slow tumor growth but tumor regrowth and spread to distant sites usually occurs after the conclusion of treatment. We recently demonstrated that macrophages could be used to deliver large quantities of a hypoxia-regulated, prostate-specific oncolytic (OV) virus to prostate tumours. In the current study we show that administration of such OV-armed macrophages 48 hours after chemotherapy (docetaxel) or tumor irradiation abolished the post-treatment regrowth of primary prostate tumours 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 to 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 prostate cancer patients.
Introduction

Solid human and murine tumors often respond well initially to conventional, front-line therapies like 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 (TAMs) and accumulate in poorly vascularised, 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 forty eight hours after chemotherapy (docetaxel) and tumour irradiation and found to halt both the regrowth and metastatic spread of human prostate tumor xenografts after these therapies.
Materials & 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 (MDMs)

Macrophages were prepared from mononuclear cells isolated from buffy coats (Blood Transfusion Service, Sheffield, UK) (3).

Co-transduction of primary MDMs

To prevent undesirable viral recombination events, the HRE-regulated E1A/B gene constructs were transferred into macrophages by plasmid transfection rather than co-infection with a second viral vector. For co-transduction, MDMs (2 x 10^6) that had been cultured for 3 days were infected with adenovirus with an MOI of 100 PFU/cell and incubated overnight and then transfected with 5 μg pcDNA3.1(+) -HRE-E1A/B (HRE-E1A/B) construct using the Amaxa Macrophage Nucleofection Kit (Amaxa Biosystems, Cologne, Germany). 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, UK). LNCap:LUC cells were obtained from Professor Magnus Essand (Upsala, Sweden) (10). The cells were cultured in RPMI-1640 supplemented with 10% FBS in a humidified, 5% CO₂ atmosphere at 37°C. Tumor cells are routinely tested for authenticity by microsatellite genotyping at the ECACC and mycoplasma testing (GENEFLOW, Elmhurst, UK).

(i) 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-labeled tumor cells, enabling real-time monitoring of tumor growth and spread in the mice. The mice were injected intraperitoneally (i.p) with 90 mg/kg D-luciferin (Caliper Life Sciences) resolved in sterile water and anaesthetized using 2.5% isoflurane (Abbott Scandinavia AB, Solna, Sweden) in 100% oxygen at 3.5 L/min (for induction) in the anaesthesia chamber of the imaging system. Mice were transferred to the dark box and isoflurane was lowered to 1.5%. Images were taken every 3 min as a sequence of 10 images for every group of mice, once a week. Automatic contour regions of interest were created, and the tumor sizes (or tumor radiance) were quantified as photons per second per square centimeter per steradian (ps⁻¹ cm⁻² sr⁻¹). Progression and spread of tumors were evaluated by the calculating the tumor radiance values from inoculated mice in each group.

(ii) Subcutaneous prostate xenograft model
LnCAP-LUC cells were mixed 1:1 with matrigel (BD Biosciences) and 2 x 10⁶ injected subcutaneously into the hind flank region. When the tumors reached 4mm in diameter, mice were given a single dose of irradiation (20gy) using an AGO HS X-ray System MP1 (Gulmay Ltd, Shepperton). Tumor size was determined using callipers.

Docetaxel studies
Two x 10⁶ LNCaP-LUC cells were mixed 1:1 in Matrigel and injected into the dorsolateral portion of the prostate gland of male CD1 athymic mice. Tumor take and size was monitored by IVIS Lumina II imaging (IVIS, Caliper Life Sciences). Mice were were injected i.p. (intra-peritoneally) with 10mg/kg DOX (Sigma-Aldrich, Dorset, UK), on day 0, 2 and 4 and on day 6 or vehicle (EtOH, Tween 20, 5% glucose at volume 1:1:8). On day 8, mice received tail vein injections of either 3 million co-transduced macrophages (Ad[I/PPT-E1A] or reporter AdCMV-GFP at MOI 100 and HRE-E1A/B), 5 x 10¹⁰ Ad[I/PPT-E1A] only or vehicle. Animals were sacrificed once tumors reached the maximum volume permitted by UK Home Office Regulations and one hour before sacrifice, mice were injected i.v. with 60mg/kg pimonidazole hydrochloride (PIMO; a nitroimidazole compound used for detecting hypoxia in
tumor sections). Excised tissues including tumors, kidney, liver, lungs and spleen, were embedded in paraffin wax for histological/immunocytochemical labelling studies.

Irradiation studies
Male CD1 athymic mice (Harlan laboratories, Bicester, UK) were injected subcutaneously this time with $2 \times 10^6$ LNCaP-LUC cells mixed 1:1 with Matrigel (BD Biosciences, Oxford, UK) into the hind flank region. This was to allow ease of access for tumor irradiation. When tumors reached 4mm in diameter, mice received a single dose of 20 Gy RT. Restraining chambers designed to expose only the flank area of mice were used to allow highly localised irradiation of tumors. These s.c. tumors grew quickly and, in order to comply with UK Home Office Regulations, were removed by day 14 due to their large size. After 48 h this was followed by tail vein injection with 100ul PBS containing either 3 million co-transduced macrophages (Ad[I/PPT-E1A] or reporter AdCMV-GFP at MOI 100 and HRE-E1A/B), $5 \times 10^{10}$ Ad[I/PPT-E1A] only or PBS alone as described by us previously (3). Tumor size was determined using callipers. Again, animals were sacrificed once tumors reached the maximum permitted volume and tumors/organ were excised and processed as above.

Histology
Five micron, paraffin wax sections from tumours and tissue were cut, de-waxed, re-hydrated and stained with haematoxylin and eosin to enable areas of tumor necrosis to be readily visualized using morphological criteria - reduced cellular density, pale cytoplasm and pyknotic nuclei or completely disrupted cells, with or without red blood cell infiltration. To detect hypoxia bound pimonidazole (PIMO) was detected in tumor sections using Hypoxyprobe™-1MAb1, a monoclonal antibody IgG1 (Millipore, Consett, UK). PIMO labelling was then quantified across whole tumor sections using a random point scoring system based on that described by Smith et al (11). Sections were also incubated with specific antibodies for target antigens; CD31 (1:100), F4/80 (1:80) (AbD Serotec), human CD68 (Dako, Ely, UK) at 1:100 and E1A at 1:50 (Millipore, UK). A biotinylated secondary antibody system was used in conjunction with a streptavidin-conjugated HRP. Peroxidase activity was localised 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 Ep-CAM using the immunohistochemistry 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 monocyte-derived macrophages (MDMs), co-transduction of primary MDMs, assessment of tumor necrosis/hypoxia, tumor metastasis and immunolabelling/analysis of CD31, F4/80, CD68, E1A & Ep-CAM in tumor sections, please see Muthana et al (2011) and Supplementary file.

**Statistical analysis**

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

**Results & Discussion**

Three intravenous injections of docetaxel (DOX) significantly (P<0.03) delayed the growth of orthotopic prostate (LNCaP-LUC) tumors until day 14 (i.e. 10 days after the last DOX injection) (Figure 1A & Suppl Figure 1A) and improved mouse survival (Figure 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 to tumors from the ‘vehicle’ group removed by day 14 due to their large size) (Suppl Figure 1B & D), as was a marked tumor infiltration by murine TAMs by day 2, which was still present at day 35 (Figure 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 DOX injections. So, this time point was
selected for a single 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 DOX by 7 days (only occurring between days 21-28) 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 (Figure 1 A&B). Both OV treatments significantly ($P<0.001$) reduced microvessel density in DOX-treated tumors (sampled at day 28 for the DOX+free OV group and day 35 for the DOX+co-transduced macrophages) compared to DOX alone group (at day 28), but failed to affect tumor hypoxia (Suppl. Figure 1 C&D). Human CD68+ macrophages were present in tumors in mice injected with either GFP-expressing or OV-bearing macrophages (Suppl. Figure 1E), and the latter resulted in significantly ($P<0.001$) more OV detection throughout tumors after DOX than in the tumors of mice injected with free OV alone+DOX (Figure 1D).

A single dose of 20Gy radiotherapy (RT) was also seen to significantly reduced the growth of LnCAP 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 (ie. transfected to express the reporter gene, GFP) (Figure 2A & Suppl Figure 2A). Thirty-five days after RT there was a small but insignificant drop in tumor microvessel density compared to 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 (Suppl. Figures 2B-D). 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 (Figure 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 DOX 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 co-transduced
macrophages significantly extended this, with no regrowth evident by 42 days after RT (i.e. the end of the experiment) (Figure 2A & Suppl. Figure 2A). This correlated with improved survival rates in the latter group, compared to those receiving RT with free OV (Figure 2B). There were also significantly ($P<0.001$) fewer CD31$^+$ blood vessels and more necrosis in tumors receiving macrophage-delivered OV than OV alone (Suppl. Figures 2B-D). Following RT, human CD68$^+$ macrophages were present in tumors receiving macrophage-delivered GFP and OV (Figure 2C & Suppl. Figure 2E), leading to widespread expression of OV in the latter group (Figure 2D).

We next determined how these therapies influenced the development of pulmonary metastases. Few metastases were detected in mice injected with PBS alone (no DOX) because, as mentioned previously, primary tumours 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 (Figure 3A). The formation of lung metastases after DOX was abolished when OV-bearing macrophages were injected 2 days after the final DOX was delivered (a phenomenon not seen when mice were injected with free OV or macrophages bearing a control, GFP-expressing adenovirus) (Figure 3A&B). OV was detected in some areas of lung in mice injected with OV-bearing macrophages after DOX, but not DOX+free OV (Figure 4A). Similarly, pulmonary metastases were significantly ($P<0.0012$) higher in mice receiving RT+free OV than RT+co-transduced macrophages (a valid comparison as both groups were sacrificed on day 42) (Figure 3C&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 (Figure 4B). 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 our co-transduced, 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 DOX study). It should be noted that the
majority of pre-clinical studies of anti-cancer gene therapies have used similar human tumour xenograft models (12, 13).

In sum, the current study shows that it is possible to exploit the increased macrophage infiltration of 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 prostate cancer patients.

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References


Figure Legends

Figure 1. Macrophage delivery of an oncolytic virus (Ad[I/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 (10mg/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 co-transduced 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 co-transduced 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) (x20 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+co-transduced macrophage group. Representative data shown for one of two replicate experiments where N=5 mice/group. Data are means ± SEM. Statistical significance differences. *P < 0.05 or **P < 0.01 compared with DOX+free OV group; †P < 0.01 compared with DOX alone. Bar = 200μM.

Figure 2. Macrophage delivery of an oncolytic virus (Ad[1/PPT-E1A]) abolishes the regrowth of prostate (LUC-LNCaP) tumors following irradiation. Tumor-bearing mice were administered a single dose of 20Gy radiotherapy (RT) and then injected intravenously 2 days later with a single dose of either the oncolytic virus (OV) alone or MDMs co-transduced with an HRE-E1/A/B plasmid and either a reporter virus AdGFP or OV. (A) Tumor luminosity showing RT alone significantly reduced tumor growth for 14 days but tumors then started to regrow by day 21. A single injection of OV alone delayed this post-RT regrowth by up to a week but viral delivery via co-transduced macrophages completely abolished it for 42 days. [Circles represent times when tumors were removed for analysis]. (B) Mouse survival [NB the final data points on each line were when mice were culled]. (C) The number of murine F4/80+ TAMs in 6 HPF per tissue section increased in all RT-treated groups except the RT+co-transduced macrophage group. (D) OV infection (viral E1A protein staining; red – see arrows) occurred in tumors after RT+OV alone but was higher in the RT+co-transduced MDMs group. Representative images of E1A positive cells at x10 and x40 magnification. Representative data shown for one of two replicate experiments where N=6 mice/group. Data are means ± SEM. Statistical significance differences *P < 0.05, **P < 0.01 compared with RT+free OV group. Bar = 100μM (x10)and 200μM (x20).

Figure 3. Macrophage delivery of an oncolytic virus (Ad[1/PPT-E1A]) abolishes the formation of pulmonary metastases in mice bearing human prostate (LNCaP-LUC) tumors after docetaxel treatment or irradiation. Representative appearance of pulmonary metastases in anti-Ep-CAM stained sections revealed very few metastases in the lungs of control tumor-bearing mice by day 14 but were evident by days 28-35 in tumors from mice receiving 3 injections of DOX (A&B) or days 35-42 in
those receiving 20 Gy RT (C&D). Quantification of metastatic foci per lung section per mouse after (B) DOX or (D) RT treatment was not affected by injection of OV alone but was virtually abolished by a single injection of OV-bearing macrophages. Of note, each lung was serially sectioned, 5 sections 100 μm apart were stained with anti-Ep-CAM, and the total number of metastatic foci (>5 cells) was quantified per mouse (n ≥5-6 mice per group). Representative data are shown for one of two replicate experiments. SEMs are depicted with *P < 0.01 compared with DOX+free OV or RT+free OV groups (as appropriate). Bar = 200μM.
Figure 1

A. Regrowth

B. Percent survival

C. No. F4/80+ cells/field

D. % ETA+ cells

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Figure 1

A. Regrowth

B. Percent survival

C. No. F4/80+ cells/field

D. % ETA+ cells
Figure 2

A

B

C

D

RT+OV

RT+MDM+OV

N

% EIA+ cells

0 20 40 60 80

RT+OV

RT+MDM+OV

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Figure 3

A

Vehicle  DOX

DOX+OV  DOX+MDM

C

PBS  RT

RT+OV  RT+MDM+OV

B

Pulmonary Metastasis

[Graph showing data for different treatments: Vehicle, DOX, DOX+OV, DOX+MDM, RT, RT+OV, RT+MDM+OV.]

D

Pulmonary Metastasis

[Graph showing data for different treatments: PBS, RT, RT+MDM+GFP, RT+OV, RT+MDM+OV.]

Note: The data points are marked with asterisks to indicate statistical significance. Legends for treatments are provided at the bottom of the graph.
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