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Research Paper

Genetically engineered oncolytic Newcastle disease virus mediates cytolysis of prostate cancer stem like cells

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ABSTRACT

Oncolytic virotherapy is a promising novel approach that overcomes the limitations posed by radiation and chemotherapy. In this study, the oncolytic efficacy of a recombinant Newcastle disease virus (rNDV) BC-KLQL-GFP, against prostate cancer stem-like/tumor initiating cells was evaluated. Xenograft derived prostaspheres (XPS) induced tumor more efficiently than monolayer cell derived prostaspheres (MCPS) in nude mice. Primary and secondary XPS show enhanced self-renewal and clonogenic potential compared to MCPS. XPS also expressed embryonic stem cell markers, such as Nanog, CD44 and Nestin. Further, prostate specific antigen (PSA) activated recombinant Newcastle Disease Virus (rNDV) was selectively cytotoxic to tumor derived DU145 prostaspheres. An effective concentration (EC_{50}) of 0.11–0.14 multiplicity of infection was sufficient to cause prostasphere cell death in serum free culture. DU145 tumor xenograft derived prostaspheres were used as tumor surrogates as they were enriched for a putative tumor initiating cell population. PSA activated rNDV was efficient in inducing cell death of cells and prostaspheres derived from primary xenografts *ex-vivo*, thus signifying a potential *in vivo* efficacy. The EC_{50} (~0.1 MOI) for cytolysis of tumor initiating cells was slightly higher than that was required for the parental cell line, but within the therapeutic margin for safety and efficacy.

1. Introduction

Prostate cancer (CaP) is the most common neoplasm affecting men and the second leading cause of cancer-related deaths in males in the developed world ("American Cancer Society: Cancer Facts and figures, 2012"; Jemal et al., 2007; Williams and Powell, 2009). Multiple cell types in the prostate gland have been implicated to be potential cells of origin for tumor development (Korsten et al., 2009; Lawson et al., 2010; Tokar et al., 2005; Wang et al., 2009). Although the mechanisms responsible for CaP remain elusive, prostate cancer stem cells (PSCs) are regarded to be the origin of CaP initiation and progression (Kasper, 2008; Lawson and Witte, 2007; Tang et al., 2007). Cancer stem cells (CSCs) that originate the tumor share surface antigens with their tissue stem cell counterparts (Al-Hajj et al., 2003; O'Brien et al., 2007; Ricci-Vitiani et al., 2007; Singh et al., 2003; Singh et al., 2004b). The CSCs are similar to normal stem cells, possess self-renewal, multipotency, relative quiescence, and cytoprotective mechanisms, including DNA repair and expression of drug transporters (Dey et al., 2011). Presence of these cytoprotective mechanisms enables CSCs to evade conventional cytotoxic therapy. Previously, many have reported that CaP cells expressing surface antigens like CD44 CD133, CD151 and CD166 are good candidates for detecting cancer stem cells (Collins et al., 2005), (Patrawala et al., 2006), (Rajasekhar et al., 2011). Others have shown that pluripotent stem cell marker TRA-1-60 and tumor protein p63 are crucial for identifying a tumor initiating cell population (Huang et al., 2015; Rajasekhar et al., 2011). However, the biggest challenge is to relate marker expression to cell lineage and identify putative CaP tumor initiating cells.

Oncolytic virotherapy offers a novel approach for eradicating CSCs, using unique mechanisms of cytolysis that differ from conventional therapies. In addition, oncolytic viruses exert their effects as a fine interplay between antitumor and antiviral immune responses (Fulci et al., 2006; Parker et al., 2000). Earlier, we have shown that prostate specific antigen (PSA) re-targeted recombinant Newcastle disease virus (rNDV), whose fusion peptide was modified from ^{114}QRR//F^{117} to ^{114}KLQ//L^{117}, was highly cytotoxic to CaP cells (Shobana et al., 2013). This prompted us to test whether PSA retargeted rNDV will be cytotoxic to CaP initiating cells. We examined DU145 prostate cancer cells and

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prostaspheres for the presence of tumor initiating cells, and tested the oncolytic efficacy of PSA targeted rNDV to primary xenograft derived DU145 prostaspheres. Our results suggest that prostaspheres are enriched in cancer stem like cells and that PSA targeted rNDV is cytotoxic to these CaP initiating cells.

2. Materials and methods

2.1. In vitro prostasphere culture

Prostaspheres were generated from the DU145 cell line as described previously (Shobana et al., 2013). Briefly, monolayer cells and primary tumor cells were digested using Hyqtase (HyClone) and resuspended in serum-free DMEM/F12 medium (Invitrogen) containing 1x glutamax (Invitrogen), 1x B27 lacking vitamin A (Invitrogen) and supplemented with recombinant epidermal growth factor (EGF) at 10 ng/ml (Invitrogen). Typical spheres formed in 7 days. Spheres were subcultured using Hyqtase, counted and resuspended in the above medium. To assay the proliferative capacity of secondary and tertiary spheres, the cells were dissociated, plated at a clonal density of 2×10^5 cells/5 ml and counted after 7 days.

2.2. Tumor xenotransplantation

Female athymic nude mice, strain Foxn1nu (Harlan Laboratories), aged 6 weeks were transplanted with 1×10^2, 1×10^4 and 1×10^6 DU145 prostaspheres (PS) or monolayer cells (Huang et al.) in 100 µl of phosphate buffered saline, into the right flank as subcutaneous tumors. Mice were inspected for tumors and the tumor size was measured every two days using a vernier caliper. The tumor volume was determined using the formula $L*W^2/2$, where L and W are the longest and the shortest diameters of the tumor mass, respectively. Tumors were harvested and the primary tumor MC and PS were subcutaneously transplanted into a new set of nude mice to produce secondary xenografts.

2.3. Prostate cancer cell lines and primary xenograft tumor derived cells

Androgen receptor negative (AR^-) DU145 cell line was purchased from ATCC and maintained in Dulbecco's modified Eagle's medium (DMEM) (Invitrogen) supplemented with 10% fetal calf serum. DU145 xenografts from BALB/c nude mice were harvested and dissociated tissue was passed through a 100 µm cell strainer (BD Falcon) to remove cell aggregates and debris. The resulting cells were pelleted at 800 g and live cell numbers were determined by trypan blue exclusion (Rajasekhar et al., 2011) and were plated to generate prostaspheres.

2.4. Soft agar clonogenicity assay

DU145 monolayer and tumor xenograft derived cells were plated in individual wells of a six-well plate at densities 4×10^2, 4×10^3 and 4×10^4 cells/well in media containing 0.3% agarose and overlaid on 0.6% agarose as previously described (Webber et al., 2001).

2.5. Cancer stem cell marker staining and cell sorting

Immunofluorescence staining of DU145 MC, primary tumor cells and PS was performed by fixing cells with methanol: acetone (1:1) solution for 10 min at −20 °C, followed by staining with the specific primary antibodies. Slides were incubated in the dark with primary antibodies for 1hr at 37 °C. Chamber slides were subsequently washed in 1 x PBST and incubated with secondary antibodies for 1 h at 37 °C. Slides were then washed and mounted with a coverslip using Vectashield mounting medium with DAPI (Vector laboratories). Images were captured using a laser-scanning microscope (Carl Zeiss LSM 510). Cells were then stained with anti-human ABCG2 antibody for 1 h at 4 °C, washed twice with PBS and sorted by fluorescence activated cell sorting.

2.6. Multi-step growth kinetics of rNDV

Prostaspheres were seeded in 6-well plates at 5×10^5 cells per well and infected with rNDV (BC-KLQL-GFP) at a multiplicity of infection (MOI) of 0.01, 0.1 and 1 for multi-step growth kinetics. Virus titers were obtained by calculating the $TCID_{50}$ using Reed and Muench method as described (Flint et al., 2009).

2.7. Cytotoxicity by trypan blue dye exclusion assay

Prostaspheres were plated as five replicates in 6-well plates at a density of 5×10^5 cells/well and infected with rNDV at 0.01, 0.1, 1.0 and 10.0 MOI. Cells were trypsinized at 24, 48, 72, 96 and 120 h post infection and cell viability was determined using the trypan blue dye exclusion assay (Altman et al., 1993; Tennant, 1964).

2.8. Statistical analysis

Statistical analysis was performed using JMP 9 for Mac software. Data were presented as mean ± standard deviation unless otherwise stated. All Student *t*-tests performed were two-tailed and a p-value < 0.05 was considered statistically significant. Power analysis was also carried out using JMP 9 to determine the accuracy of replicates for a given experiment. The 50% effective concentration values were calculated using the dose versus inhibitor response curve with four parameters using Graphpad prism software (Graph Pad Inc.).

3. Results

Recently, we described that PSA targeted rNDV is capable of lysing CaP cells and cell line derived spheres in a PSA dependent manner (Shobana et al., 2013). In order to test whether this rNDV would also be able to target and lyse CaP initiating cells, we characterized stem-like population from an androgen receptor (AR) negative, androgen independent human DU145 cell line and tumor xenograft derived prostaspheres (PS). A 100 ng/ml of prostate specific antigen overlay was used to activate PSA targeted NDV infection in DU145 monolayer cells and xenograft derived cells.

3.1. Xenograft derived prostaspheres (XPS) induces tumor more efficiently than monolayer cell derived prostaspheres (MCPS) in nude mice

To evaluate tumorigenic potential, 10^2, 10^4 and 10^6 of either MCPS or MC were subcutaneously implanted in athymic nude mice. We also sorted DU145 MCs for the known cancer stem cell marker, ABCG2 (23) and subsequently implanted the $ABCG2^-$ population cells in athymic nude mice. Table 1 shows three types of cells used to generate primary tumor xenografts. The number of mice that developed palpable tumors and the latency associated with tumor development were documented. One out of ten mice implanted with 10^4 DU145 MCPS developed a tumor in 37 days, and one out of five mice injected with 10^6 MCPS developed a tumor in 30 days. No tumors could be detected in the groups that received DU145 MC even after 60 days post implantation and thus monolayer cells served as a negative control throughout the study. Interestingly, two out of ten mice that received 10^4 DU145 $ABCG2^-$ cells also formed tumors at 35 and 50 days respectively. To further assess the ability of PS to form secondary tumors, DU145 MCPS tumor xenografts were harvested and cultured as cells or spheres for a single passage, and were then implanted subcutaneously into athymic female nude mice (Table 1, secondary xenografts). We found that all mice that received 10^6 cells developed palpable tumors, whether they were cultured as primary tumor cells or as spheres.