NOVA

University of Newcastle Research Online

nova.newcastle.edu.au

Skelding, Kathryn A.; Barry, Richard D.; Shafren, Darren R. "Systemic targeting of metastatic human breast tumor xenografts by Cocksackievirus A21". Originally published Breast Cancer Research and Treatment Vol. 113, Issue 1, p. 21-30

Available from: <http://dx.doi.org/10.1007/s10549-008-9899-2>

The original article is available at www.springerlink.com

Accessed from: <http://hdl.handle.net/1959.13/924955>

Systemic Targeting of Metastatic Human Breast Tumor Xenografts by Coxsackievirus A21

Kathryn A. Skelding¹, Richard D. Barry¹, Darren R. Shafren^{1, 2, *}

¹ The Picornavirus Research Unit, School of Biomedical Sciences, Faculty of Health, The University of Newcastle, Newcastle, New South Wales, 2300, Australia.

² Viralytics Ltd, Unit 8, 33 Ryde Rd, Pymble, New South Wales, 2073, Australia.

Disclosure: Darren R. Shafren is a director of Viralytics Ltd. Richard D. Barry and Darren R. Shafren hold shares in Viralytics Ltd and are currently conducting research sponsored by this company.

*Corresponding author:

Darren Shafren

The Picornaviral Research Unit, Discipline of Immunology and Microbiology, Faculty of Health, The University of Newcastle, David Maddison Clinical Sciences Building, Newcastle, New South Wales, 2300, Australia

Phone: +61-2-4923-6158

Fax: +61-2-4923-6814

E-mail: dshafren@mail.newcastle.edu.au

Running Title: Systemic CVA21 therapy as a treatment for metastatic breast cancer

Article Type: Preclinical Study

Keywords: breast cancer, Coxsackievirus A21, oncolytic virus, virotherapy

ABSTRACT

Breast cancer is the most commonly diagnosed malignancy in women worldwide. Metastatic development is associated with poor prognosis and current therapies provide only limited success. Virotherapy is an emerging strategy for the treatment of cancer that utilizes both replication-competent and genetically modified viruses to selectively kill tumor cells. We have previously shown that Coxsackievirus A21 (CVA21), a wild-type common-cold producing enterovirus, is an effective oncolytic agent against human melanoma xenografts *in vivo*. CVA21 specifically targets and lytically infects susceptible cells expressing the CVA21 cellular receptors, intercellular adhesion molecule-1 (ICAM-1) and/or decay-accelerating factor (DAF). Herein, the efficacy of CVA21 as a therapeutic agent against human breast cancer was investigated both *in vitro* and *in vivo*. Flow cytometric analysis revealed that the human breast cancer cell lines examined expressed significantly elevated levels of surface ICAM-1 and DAF compared to normal breast cell lines, and that all cancerous lines were more susceptible to lytic infection by CVA21 than the normal cells. Through the use of subcutaneous (T47D cells) and orthotopic (MDA-MB-231-luc cells) xenograft SCID mouse models it was demonstrated that a single intravenous injection of CVA21 produced significant regression of pre-established tumors *in vivo*, as well as targeting and elimination of metastases in the orthotopic model. Taken together, these findings highlight the exciting potential of CVA21 as a therapeutic agent against both primary and metastatic human breast cancer.

INTRODUCTION

Breast cancer is the second most frequent cancer in the world, with more than 1.2 million women estimated to be diagnosed each year [1]. Conventional therapies, including surgery and radiation therapy, have yielded rather limited success in the treatment of human breast cancer following metastatic spread [2], highlighting the need for the continuing development of new therapeutics. Viral oncolysis has recently emerged as a potential novel anti-cancer treatment that involves the use of naturally occurring or genetically modified viruses to selectively target and lyse neoplastic cells [3-5]. One such oncolytic virus is the wild-type genetically unmodified human enterovirus, Coxsackievirus A21 (CVA21), a benign virus which induces mild upper respiratory symptoms during natural infection of humans [6-8]. CVA21 naturally infects cells in the respiratory tract, which are known to express ICAM-1 [9, 10], thereby resulting in the development of ‘common cold’ like symptoms commonly observed during natural and experimental infection.

The cellular receptor complex utilized by CVA21 consists of intercellular adhesion molecule-1 (ICAM-1) and decay-accelerating factor (DAF) [11, 12]. In this receptor complex, DAF functions as a ‘concentration’ receptor; viral interactions with DAF alone are not able to induce lytic infection by the *Kuykendall* prototype strain of CVA21. Rather, viral binding to DAF aids in the capture and presentation of virus for subsequent interaction with the internalization receptor, ICAM-1 [12, 13], which leads to cellular entry and consequent lytic infection. Progeny virus released from infected cells is then capable of infecting adjacent cells, entering the systemic circulation, and potentially targeting micrometastatic foci. CVA21 is an efficient oncolytic agent which specifically targets and rapidly lyses human malignant

melanoma tumors expressing upregulated levels of the CVA21 cellular receptors both *in vitro* and *in vivo* [14, 15]. In more recent studies, CVA21 exhibited significant oncolytic activity against multiple myeloma cells and to have a potential role in the *ex vivo* purging of malignant plasma cells prior to autologous stem cell transplantation [16].

In general, when compared to the surrounding normal tissue, ICAM-1 and DAF are over-expressed on many cancers, including breast tumors. ICAM-1, an immunoglobulin-like molecule, is highly expressed on the surface of primary human breast cancers [17]. ICAM-1 expression has also recently been linked to the invasiveness of breast cancer cells *in vitro* [18]. Elevated surface expression of ICAM-1 is hypothesized to enhance the potential for tumor cells to interact with circulating lymphocytes via interactions with the integrin molecule lymphocyte function associated antigen-1 (LFA-1), thus potentially providing a means by which cancer cells undergo metastatic spread [15]. The complement regulatory protein, DAF, is also over-expressed on tumor cells, including less aggressive breast tumors [19], and functions to protect tumor cells from complement-mediated cytotoxicity [20].

In this study we demonstrate that CVA21 lytically infects and replicates in a panel of human breast tumor-derived cell lines that express the cellular receptors, ICAM-1 and DAF, to a greater extent than cells derived from normal breast tissue. The oncolytic action of CVA21 was confirmed *in vivo*, as evidenced by regression of primary tumors in two human breast xenograft models and elimination of metastases in an orthotopic model in SCID mice following systemic viral administration. The above

findings highlight the potential application of a genetically unmodified wild-type strain of CVA21 as an effective bio-therapeutic agent for the treatment of both primary and metastatic breast cancers.

MATERIALS AND METHODS

Cells and viruses. The breast cancer cell lines BT-20, MDA-MB-157, MDA-MB-453, SK-BR-3, and ZR-75-1, and the normal cell line MCF-10A, were obtained from Dr. I. Campbell (Peter MacCallum Cancer Centre, Melbourne, Australia), and the MDA-MB-231, MDA-MB-361, MCF-7, and T-47D cells were obtained from Dr. R. Sutherland (Garvan Institute of Medical Research, Sydney, Australia). The luciferase expressing MDA-MB-231-luc were obtained from Dr. J. Shay (University of Texas Southwestern Medical Centre, Dallas, USA). The normal breast cell lines, 184A1 and Hs578Bst, were purchased from the American Tissue Culture Collection. Melanoma SK-Mel-28 cells were obtained from Dr. S. Ralph (Department of Biochemistry and Molecular Biology, Monash University, Victoria, Australia). All of the breast cancer cell lines, as well as the MCF-10A line, were maintained in RPMI 1640 (Invitrogen, Auckland, NZ) supplemented with 10% fetal bovine serum (FBS) (Invitrogen), whilst SK-Mel-28 cells were maintained in DMEM (Invitrogen), supplemented with 2% FBS. 184A1 and Hs578Bst cells were maintained in MEGM (Cambrex, Mt Waverly, Australia) supplemented with 10% FBS, bovine pituitary extract, hydrocortisone, human epidermal growth factor, and insulin, as per manufacturer's instructions.

The *Kuykendall* prototype strain of Cocksackievirus A21 was obtained from Dr. M. Kennett (Entero-respiratory Laboratory, Fairfield Hospital) and was propagated in SK-Mel-28 cells. Stock preparations of CVA21 were purified by centrifugation in 5-30% sucrose gradients as described previously [21]. The peak infectious fractions were pooled, dialyzed against PBS, and stored at -80°C.

Antibodies. Anti-DAF monoclonal antibody, IH4, which recognizes the third short consensus repeat of DAF [22], was a gift from Dr. B. Loveland (Austin Research Institute, Melbourne, Australia). The anti-ICAM-1 monoclonal antibody, WEHI, specific for the N-terminal domain of ICAM-1 [23], was obtained from Dr. A. Boyd (Queensland Institute for Medical Research, Brisbane, Australia).

Flow cytometry. The surface expression of DAF and ICAM-1 on breast cell lines was determined by flow cytometry. Briefly, 1×10^6 cells were pelleted at 1000 x g for 5 min and incubated on ice with anti-ICAM-1 or anti-DAF monoclonal antibodies (5 µg/ml diluted in PBS) for 20 min. Cells were then washed with PBS, pelleted at 1000 x g for 5 min, and resuspended in 100 µl of R-phycoerythrin-conjugated F(ab')₂ fragment of goat anti-mouse immunoglobulin (DAKO A/S) diluted 1:100 in PBS. Following incubation on ice for 20 min, cells were washed and pelleted as above, resuspended in PBS and analyzed for DAF and ICAM-1 expression using a FACScan analyser (Becton Dickinson, Sydney, Australia).

***In vitro* viral infectivity assay.** Confluent monolayers of breast cell lines in 96-well tissue culture plates were infected with 10-fold serial CVA21 dilutions of stock preparations (100 µl/well in quadruplicate) that were standardized on SK-Mel-28 cells. Following incubation at 37°C for 72 h, cell monolayers were microscopically examined for cellular lysis and fifty percent tissue culture infectious doses (TCID₅₀) were calculated using the method of Karber [24].

***In vitro* viral growth assay in infected cell lines.** Confluent cell monolayers in 24-well culture plates were inoculated with CVA21 at a multiplicity of infection

(MOI) of 2 TCID₅₀/cell in RPMI 1640 supplemented with 1% FBS, and incubated at 37°C for 1 h. Unbound virus was removed, the cells washed 3 times and overlaid with maintenance media. Following incubation for 0, 24, and 48 h at 37°C, cell suspensions were freeze/thawed once, and the level of infectious CVA21 in cells and supernatant was determined by a microtiter plate cell lysis infectivity assay as described above.

Infection of three-dimensional spheroid cultures of cancer cells. MDA-MB-231 cells were seeded into a 24-well plate at cell numbers ranging from 5×10^3 to 9×10^4 cells/well in 1 ml of RPMI-1640 containing 5% FBS, and overlaid onto a semi-solid 0.5% agarose layer. Cells were allowed to form spheroids for 48 h at 37°C in a 5% CO₂ atmosphere. CVA21 was added (3×10^6 TCID₅₀), and the destruction of the pre-formed spheroids was monitored daily over a 12-day period.

Subcutaneous breast tumor xenograft transplantation in severe combined immunodeficient (SCID) mice. All animal procedures were performed under the guidance and approval of the University of Newcastle Animal Care and Ethics Committee. Specific-pathogen-free (SPF) SCID mice were housed under pathogen-free conditions in the animal handling facility located within the university. Breast cells (T47D) were harvested with trypsin, washed once with RPMI 1640, 3 times with PBS and resuspended in sterile PBS. The viability of cells was assessed by trypan blue staining and only cell preparations with >95% viability were used for xenotransplantation. Tumor cells were injected onto the flanks of anaesthetized (isoflurane) 6 to 8 week old SCID mice by single subcutaneous injection of 4×10^6 cells. Once palpable tumors had established, the tumor bearing mice (5 per group)

were treated with a single intravenous injection of PBS or CVA21 (5×10^7 TCID₅₀). Xenograft tumor burdens were monitored daily and measured with electronic callipers. Estimates of tumor volumes were calculated using the formula for a spheroid.

Orthotopic breast tumor xenograft transplantation in severe combined immunodeficient (SCID) mice. Prior to injection, breast cancer cells (MDA-MB-231-luc) were examined for luciferase expression *in vitro* using an IVISTM Imaging System (Xenogen, California, USA), and acquired and analysed using Living Image® software (Xenogen). Briefly, cells were diluted from 100,000 to 1 cell into appropriate cell culture media in black 96-well plates. Twenty microliters of 1x lysis buffer (Promega, Sydney, Australia) was added to each well, and incubated at room temperature for 5 min to ensure complete cellular lysis. Next, 50 µl of luciferase reagent (Promega) was added to each well, and the luminescence was immediately examined using the IVISTM Imaging System. Once luciferase expression was confirmed, breast cancer cells (MDA-MB-231-luc) were harvested as described above, before being implanted onto the fourth right mammary fat pad of anaesthetized (isofluorane) 6 to 8 week old SCID mice by single subcutaneous injection of 2×10^6 cells. Once palpable tumors had established, the tumor bearing mice (8 per group) were treated with a single intravenous injection of PBS or CVA21 (1×10^7 TCID₅₀). Xenograft tumor burdens were monitored daily and measured with electronic callipers, and estimates of tumor volumes calculated using the formula for a spheroid. *In vivo* bioluminescent imaging was performed using an IVISTM Imaging. Animals were administered D-luciferin (Xenogen) intraperitoneally at 5 mg/mouse in PBS, and then anaesthetized using isofluorane. At 5 – 10 min post luciferin injection, mice

were placed into the IVISTM Imaging System and imaged for 1 s – 2 min. The lower half of each animal was shielded (in order to cover the primary tumor and reduce luminescence from this tumor), and the mouse was then re-imaged to detect the presence of any metastases in the lungs or lymph nodes. Mice were monitored over a 42-day period, at which time, the PBS-treated control mice were sacrificed due to excessive tumor burden.

Statistical analysis. Receptor expression comparison analyses were conducted using a Wilcoxon signed-rank test. All animal experiment statistical analyses were conducted using a non-parametric t-test, and were presented as the mean $\pm$ standard error of the mean (SEM) for the number of mice in each group (n).

RESULTS

Expression of ICAM-1 and DAF on Breast Cell Lines

CVA21 is able to target and rapidly lyse human malignant melanoma cells expressing high levels of the CVA21 cellular receptors, ICAM-1 and DAF [15]. Herein, the cell surface expression of these receptors was examined in a panel of normal and cancerous breast cell lines. Flow cytometric analysis revealed that 7 of the 9 cancer cell lines examined possessed abundant levels of surface ICAM-1 and DAF, whilst the ZR-75-1 and SK-BR-3 neoplastic cell lines expressed moderate levels, and the normal cell lines (184A1, Hs578Bst, and MCF-10A) only expressed minimal surface ICAM-1 (Figure 1). Most cell lines examined expressed high levels of surface DAF, except for Hs578Bst, which expressed only minimal levels (Figure 1). Furthermore, the expression of ICAM-1 and DAF on cancer cells was found to be significantly higher ($p = 0.004$ and $p = 0.02$, respectively) than that observed on the normal breast cell lines.

In vitro Lytic Infection of Human Breast Cells by CVA21

Having established elevated levels of DAF and ICAM-1 on breast cancer cells, we next investigated the oncolytic capacity of CVA21 on both normal and cancerous *in vitro* breast cell cultures. Cell monolayers were infected with CVA21 at varying input multiplicities of infection (MOI) and incubated at 37°C for 72 hours. Microscopic examination revealed that CVA21 infection at a MOI of 100 induced extensive lytic destruction of all of the cancer cell lines, whilst the normal cells were relatively untouched (Figure 2A). Quantification of CVA21 mediated cell lysis revealed that at least 8 of the 9 cancer cell lines were lytically infected to a greater extent than the normalized cell lines 184A1 and MCF-10A (Figure 2B). The majority

of the breast cancer cell lines were lysed at CVA21 MOIs less than or equal to 1, whereas the normal lines required a viral input dose approximately 3 to 1000-fold greater to be lytically infected.

The production of progeny virus by replication-competent oncolytic viruses provides a major advantage over the use of replication-deficient oncolytic viruses. The *in vivo* amplification of the viral input dose facilitates infection of adjacent cells and thus allows potential viral spread through systemic circulation to distant sites of metastasis [25]. To assess the level of production of CVA21 progeny virus in human breast cell lines, both normal and cancer cell lines were challenged with CVA21 (MOI = 2 TCID₅₀/cell), and the production of progeny virus was monitored over a 48 h period. As predicted by the minimal ICAM-1 expression, lower levels of CVA21 attached to the normal cells compared to the higher ICAM-1 expressing cancer cells immediately following viral challenge (Figure 3). CVA21 underwent multi-cycle replication to varying degrees in all of the cell lines, with the cancer cell lines in general producing a higher viral yield (0.5 – 4 log₁₀ greater) than the normal line (Figure 3). At a viral input dose of 2 TCID₅₀/cell, no detectable cytopathic effects were observed in the normal line at 48 h post-infection. In contrast, at this challenge dose, CVA21 induced extensive levels of cell cytolysis in all of the breast cancer cell lines. However, despite the lack of detectable cytopathic changes in the normal breast line at this viral input dose, low levels of viral replication were observed. Previously, in the absence of detectable cell cytopathology, a low level of CVA21 replication was observed in RD cells that express DAF, but not ICAM-1 [26]. The apparent low level of viral replication in the normal and RD cells may be due to CVA21 binding to DAF, and

eluting off as infectious particles, or undergoing low level replication following cell entry via a DAF dependent mechanism, possibly via a caveolae-dependent route [27].

In an attempt to provide a more rigorous challenge and to produce an environment closer to that of *in vivo* solid tumors, breast cancer cells were propagated as three-dimensional spheroid cultures, and challenged with CVA21. Spheroids more closely mimic tumors at their initial stages, and represent micrometastatic foci [28]. MDA-MB-231 cells were seeded at various concentrations on semi-solid agarose/media suspension, and were allowed to form three-dimensional cell aggregates for 48 h before being challenged with 3×10^6 TCID₅₀ of CVA21 or media alone. Virus treated spheroids displayed extensive lytic destruction, whilst mock-infected spheroids continued to increase or stabilize in volume over this period (Figure 4). Successful destruction of the breast cancer cell aggregates suggests that CVA21 could potentially be utilized to lyse solid tumors or micrometastatic foci *in vivo*.

In Vivo Oncolysis of Human Breast Cancer Xenografts by CVA21

The *in vitro* data presented herein indicates that CVA21 can efficiently lyse human breast cancer cell lines expressing high levels of ICAM-1 and DAF. To assess if the *in vitro* susceptibility of the cell lines is a predictive indicator of CVA21 oncolytic effect *in vivo*, a series of animal challenge experiments were performed. SCID mice with pre-established T47D subcutaneous tumor xenografts (~20 mm³ in size) were treated with a single intravenous injection of either PBS or CVA21 (5×10^7 TCID₅₀), and their tumor burdens were measured by callipers over a 26-day period (Figure 5). CVA21 administration significantly ($p < 0.05$) reduced the tumor burden of these mice when compared to PBS treated mice as early as 7 days following CVA21

administration. All of the CVA21 treated mice responded to virus treatment, characterised by significant tumor regression. On the day of sacrifice, tumors from CVA21 challenged mice were found to have regressed to between 3 and 30% of their original size (average $17 \pm 6\%$), whereas the PBS treated tumors were found to have increased 300 to 5000% of their original size (average $2422 \pm 932\%$).

Next we investigated the oncolytic potential of CVA21 in an orthotopic model of breast cancer that results in spontaneous metastases to the lymph nodes and lungs. Breast cancer cells stably expressing the luciferase gene (MDA-MB-231-luc) were utilized for this model, as they allowed *in vivo* bioluminescent imaging of tumor development. MDA-MB-231-luc cells were serially diluted into a 96-well black plate and submitted to bioluminescent imaging, with the minimum number of detectable cells found to be 10^2 (Figure 6B). Following the display of susceptibility to challenge with CVA21, MDA-MB-231-luc cells were used to establish an orthotopic model of metastatic human breast cancer. Firstly, SCID mice with pre-established MDA-MB-231-luc tumors ($10 - 50 \text{ mm}^3$ in size) in their fourth right mammary fat pads were treated with a single intravenous injection of either PBS or CVA21 ($1 \times 10^7 \text{ TCID}_{50}$), and their primary tumor burdens were measured using callipers over a 42-day period (Figure 6A). CVA21 administration significantly ($p < 0.04$) reduced the primary tumor burden of these mice when compared to PBS treated mice from day 14 post viral challenge. As internal metastases were not accessible for calliper measurement, non-invasive *in vivo* bioluminescent imaging was performed using an IVISTM Imaging System. Tumor development was expressed as relative luminescence (photons/sec) and was found to be comparative to primary tumor volume as measured by callipers (data not shown). All control mice administered tumor cells in the

mammary fat pad possessed metastases by six weeks post tumor cell inoculation, with the majority of mice exhibiting evidence of metastases from three weeks post tumor injection (Figure 6C). Bioluminescent images revealed that CVA21 treated mice had significantly smaller metastases present compared to the PBS control mice from day 28 post-treatment onwards ($p < 0.03$) (Figure 6D). All CVA21 treated mice were free of detectable metastases at day 42 post-treatment (Figures 6C and 6D), at which time the PBS treated mice were sacrificed due to excessive tumor burden. A slight drop in the average primary tumor volume of PBS treated mice between days 35 and 42 post-treatment was observed, as a result of several mice being sacrificed in this time period due to excessive tumor burden.

Serum viral loads for the CVA21 treated mice ranged from approximately 10^5 to 10^7 TCID₅₀/ml throughout the duration of these experiments, as determined by viral infectivity assays (data not shown). The capacity of CVA21 to induce regression of both primary and metastatic cancerous cells in SCID mice highlights its potential as a systemic therapy for metastatic breast cancer.

DISCUSSION

Metastatic breast cancer is currently considered to be incurable, with existing treatments, such as chemotherapy and radiotherapy, producing numerous undesirable side effects. In this study, CVA21 was identified as a selective anti-cancer agent against breast cancer cells via the targeting of elevated levels of the CVA21 viral receptors, ICAM-1 and DAF. Receptor targeting of breast cancer cells may offer many advantages over current anti-neoplastic regimens, which in general, lack high levels of specificity for metastatic tissue [2, 29, 30]. Rapid destruction of both *in vitro* breast cancer cells, *in vivo* breast xenografts and their spontaneous metastases in SCID mice following a single systemic dose of CVA21 further highlights the potential application of CVA21 as a therapeutic anti-cancer agent.

The CVA21 cellular receptors, ICAM-1 and DAF, are upregulated on breast cancer cells when compared to the surrounding tissue [17, 19]. Breast cancer cell lines (Figure 1) express ICAM-1 and DAF, with their surface expression being significantly higher ($p < 0.004$) than that observed on the normal lines. The selective nature of the expression of ICAM-1 and DAF between normal and neoplastic tissue offered a real potential for enhanced specificity of cancerous cell cytolysis by multi-cycle CVA21 replication.

This postulate was confirmed when CVA21 induced rapid lysis in cancer cells to a greater extent than that observed in normal breast cell lines, in both *in vitro* monolayer (Figure 2) and three-dimensional tumor spheroid (Figure 4) cultures.

One of the major findings of this study was that systemic administration of the virus led to rapid reduction of pre-established breast xenograft tumors in two distinct SCID mouse models of human breast cancer (Figures 5 and 6A), as well as the complete elimination of metastases in a spontaneous metastatic model of human breast cancer (Figures 6C and 6D). Contrasting xenograft models of human breast cancer were employed in this study; subcutaneous inoculation onto the hind flank (the T47D model), and an orthotopic model that resulted in the spontaneous formation of metastases in the lymph nodes and lungs of the mice (the MDA-MB-231-luc model). Xenograft models of human cancers often involve the subcutaneous administration of cells, therefore resulting in the formation of tumors at an anatomically inappropriate site. Similarly, metastatic models often employ an intravenous injection of the cells, which does not allow the spontaneous formation of secondary tumors from a primary nodule. Neither of these models accurately mimic the organ-specific physiological characteristics or metastatic development cascade that was observed in the orthotopic model with spontaneous formation of metastases utilized in this study. Therefore, CVA21 not only demonstrated the capacity to induce significant tumor reduction in SCID mouse xenograft models of human breast cancer, but further displayed the potential to provoke tumor reduction in an anatomically appropriate site resulting in the elimination of metastases that spontaneously arose from a primary tumor.

Interestingly, the SCID mice used in this study were found to persistently be viremic throughout this study (data not shown). Despite displaying little tumour burden, some CVA21 challenged mice were viremic (data not shown). Possible explanations for this finding are (i) continued CVA21 replication in undetected tumour masses, (ii) the inability of the mice to clear the virus due to the lack of a functional immune system

or (iii) viral replication in some group of muscle cells within the mice.” Coxsackie A group viruses were originally propagated in suckling mice that possessed an immature immune system [31, 32], and are known to replicate in the muscles of such mice [33], leading to the development of myositis and hind-limb paralysis[33]. In our laboratory, a low incidence of localised myositis in the hind limbs of severe combined immune-deficient mice persistently infected with CVA21 is occasionally observed and varies between different batches of SCID mice.

We have previously demonstrated that a single intratumoral injection of CVA21 in preformed melanoma xenografts in a SCID model caused regression in contralateral tumors [15], thereby indicating the potential application of wild-type CVA21 as a systemic oncolytic agent. However, this is the first report of the oncolytic capacity of CVA21 being assessed in a metastatic xenograft mouse model. Systemic administration of oncolytic viruses has many benefits over intralesional injections, one being the targeting and destruction of non-accessible or non-operational nodules or micrometastatic foci [34]. Breast tumor lesions, and in particular, metastatic deposits are not always accessible for intratumoral injection, therefore highlighting the need for systemic administration as a treatment for this cancer.

Many diverse oncolytic viruses, such as herpes simplex virus (HSV-1) [35], adenovirus [36], reovirus [37], and vesicular stomatitis virus [38], exhibit oncolytic activity in a wide range of tumors. However, a number of these viruses are genetically modified, which in itself raises several potential questions in the minds of genetic advisors and human ethics committees. CVA21, in contrast, is a genetically unmodified, naturally occurring virus, which causes a mild ‘common-cold’ like

syndrome following experimental intranasal challenge, which is the natural route of infection [39, 40]. Utilization of a genetically unmodified virus reduces the risk of the virus reverting to an original virulent phenotype, or producing non-specific replication or chromosomal insertion due to the addition of the genetic inserts. One benefit of using CVA21 over other oncolytic viruses is the low prevalence of anti-CVA21 neutralizing antibodies in the general population (approximately 25%) [41]. In current studies being performed in our laboratory, approximately 10 – 15% of late-stage melanoma patients exhibit antibodies against CVA21 (Shafren, unpublished data). In contrast, other commonly examined oncolytic viruses possess much higher levels of circulating antibodies in humans. For example, 37% of the United States and ~80% of the African populations possess antibodies against Adenovirus type 5 [42], 60% exhibit anti-HSV-1 antibodies [43], and approximately 50% of the human population display reovirus-specific antibodies [44, 45], compared to between 18 and 36% of people in Great Britain who possess CVA21 antibodies [41].

In conclusion, we have demonstrated the capacity of CVA21 to effectively target and lyse human breast cancer cells both *in vitro* and in mouse xenograft models. Furthermore, we have demonstrated that CVA21 can target tumor masses when administered intravenously, thereby highlighting its potential use as a systemic therapy. Perhaps more exciting was the observation that following systemic treatment with this oncolytic virus, metastases in a spontaneous metastatic model of human breast cancer were completely eliminated. Non-genetically modified wild-type CVA21 has several major advantages as an oncolytic agent, including its mild pathogenicity, rapid efficient lytic infection of cancer cells, the capacity to amplify low viral input to levels that facilitate the potential to spread to distant sites, and a low

prevalence of neutralizing antibodies present in the community. Taken together, these data indicate that CVA21 virotherapy is a promising new approach for the treatment of metastatic breast cancer.

ACKNOWLEDGEMENTS

We gratefully acknowledge those investigators mentioned in the text for the provision of monoclonal antibodies, cell lines and virus that enabled this study to be undertaken.

We would like to thank Dr Gough Au, Dr Susanne Johansson, Linda Berry, Catherine Delahunty, Erin Haley, and Debbie Pepperall for scientific advice and technical assistance throughout this project. Funding for this work was derived from grants received from the Biotechnology Innovation Fund, Hunter Medical Research Institute, and Viralytics Ltd. Kathryn Skelding is a National Breast Cancer Foundation Scholar.

Figure 1. Expression of ICAM-1 and DAF on breast cell lines and tissue. Breast cell lines were incubated with either anti-DAF monoclonal antibody (IH4), anti-ICAM-1 monoclonal antibody (WEHI), or conjugate alone. The solid histograms represent the binding of the conjugate; the grey solid line histograms represent ICAM-1 expression; and the dotted line histograms represent DAF expression.

Figure 2. Quantification of lytic infection of human breast cell lines *in vitro* by CVA21. (A) Monolayers of breast cell lines in 96-well plates were infected with 10-fold dilutions of a stock preparation of CVA21. Following incubation at 37°C for 72 h, the cell monolayers were examined microscopically for signs of cell lysis. Photomicrographs of the cytopathic effects were taken at an MOI = 100 TCID₅₀/cell. (B) Fifty percent viral endpoint titers were calculated for each cell line using the method of Karber, and multiplicities of infection (MOI) for each cell line were calculated.

Figure 3. CVA21 progeny virus production in human breast cell lines. Monolayers of breast cell lines were inoculated with CVA21 (MOI = 2 TCID₅₀/cell). Following the removal of unbound virus, the cells were incubated at 37°C for 48 h. At selected time points (0, 24, and 48 h), cell suspensions were freeze-thawed, clarified, and virus yield was determined by viral lytic assay on SK-Mel-28 cells.

Figure 4. Lysis of three-dimensional spheroid cultures of MDA-MB-231 by CVA21. MDA-MB-231 cells (9×10^4) were overlaid on 0.5% semisolid agar and allowed to form spheroids over 48 h, before being infected with 3×10^6 TCID₅₀ of

CVA21. Lysis of the spheroids was microscopically monitored over a 12-day period. Photomicrographs were taken at days 0, 5, 8, and 12.

Figure 5. Oncolytic activity of CVA21 on preformed T47D xenografts. SCID mice bearing pre-established subcutaneous T47D breast cancer tumors (~20 mm³) were treated with a single intravenous injection of either PBS or CVA21 (5×10^7 TCID₅₀). Tumors were measured via callipers, and the volume was calculated using the formula for a spheroid. All tumor volumes are expressed as the average tumor burdens $\pm$ SEM (standard error of the mean, n = 5). * statistical significance (p < 0.05).

Figure 6. Oncolytic activity of CVA21 in a spontaneous metastatic model of human breast cancer. (A) SCID mice were injected into the mammary fat pad with MDA-MB-231-luc cells (2×10^6 cells), and palpable tumors were allowed to form (~10 to 50 mm³) before the mice were treated with a single intravenous injection of either PBS or CVA21 (1×10^7 TCID₅₀). All tumor volumes are expressed as the average tumor burdens $\pm$ SEM (standard error of the mean, n = 8). * statistical significance (* p = 0.04; ** p < 0.0001). (B) Non-infected MDA-MB-231-luc cells were examined *in vitro* for bioluminescence to determine the cell number threshold for detection. Cells were plated at varying numbers ($10^5 - 1$) in black 96-well plates, lysed, and luciferin detection reagent was added to the wells. The plate was imaged for between 1 and 30 sec. Bioluminescence/well was quantified in photons/sec. (C) Metastases were detected in mice from three weeks post-cell injection onwards. The mice displayed are representative for bioluminescent observation at day 42 post treatment. (D) Measurement of bioluminescent spontaneous metastases

(photons/sec) from animals treated with either PBS or CVA21. Day 14 was the first imaging time point where metastases were detected. All measurements are expressed as the average luminescence $\pm$ SEM (n = 8). * statistical significance ($p < 0.05$); ** = no bioluminescence detected.

REFERENCES

1. Parkin DM (2001) **Global cancer statistics in the year 2000**. *Lancet Oncol* **2**:533-543.
2. Greenberg PA, Hortobagyi GN, Smith TL, Ziegler LD, et al. (1996) **Long-term follow-up of patients with complete remission following combination chemotherapy for metastatic breast cancer**. *J Clin Oncol* **14**:2197-2205.
3. Hirasawa K, Nishikawa SG, Norman KL, Alain T, et al. (2002) **Oncolytic reovirus against ovarian and colon cancer**. *Cancer Res* **62**:1696-1701.
4. Pecora AL, Rizvi N, Cohen GI, Meropol NJ, et al. (2002) **Phase I trial of intravenous administration of PV701, an oncolytic virus, in patients with advanced solid cancers**. *J Clin Oncol* **20**:2251-2266.
5. Stojdl DF, Lichty B, Knowles S, Marius R, et al. (2000) **Exploiting tumor-specific defects in the interferon pathway with a previously unknown oncolytic virus**. *Nat Med* **6**:821-825.
6. Buckland FE, Bynoe ML, and Tyrrell DA (1965) **Experiments on the spread of colds. II. Studies in volunteers with coxsackievirus A21**. *J Hyg (Lond)* **63**:327-343.
7. Couch RB, Cate TR, Gerone PJ, Fleet WF, et al. (1965) **Production of illness with a Small-Particle Aerosol of Coxsackie A21**. *J Clin Invest* **44**:535-542.
8. Spickard A, Evans H, Knight V, and Johnson K (1963) **Acute respiratory disease in normal volunteers associated with Coxsackie A-21 viral infection. III. Response to nasopharyngeal and enteric inoculation**. *J Clin Invest* **42**:840-852.
9. Pathmanathan S, Krishna MT, Blomberg A, Helleday R, et al. (2003) **Repeated daily exposure to 2 ppm nitrogen dioxide upregulates the expression of IL-5, IL-10, IL-13, and ICAM-1 in the bronchial epithelium of healthy human airways**. *Occup Environ Med* **60**:892 - 896.
10. Bianco A, Whiteman SC, Sethi SK, Allen JT, et al. (2000) **Expression of intercellular adhesion molecule-1 (ICAM-1) in nasal epithelial cells of atopic subjects: a mechanism for increased rhinovirus infection?** *Clin Exp Immunol* **121**:339 - 345.
11. Shafren DR, Dorahy DJ, Greive SJ, Burns GF, et al. (1997) **Mouse cells expressing human intercellular adhesion molecule-1 are susceptible to infection by coxsackievirus A21**. *J Virol* **71**:785-789.
12. Shafren DR, Dorahy DJ, Ingham RA, Burns GF, et al. (1997) **Coxsackievirus A21 binds to decay-accelerating factor but requires intercellular adhesion molecule 1 for cell entry**. *J Virol* **71**:4736-4743.
13. Newcombe NG, Beagley LG, Christiansen D, Loveland BE, et al. (2004) **Novel role for decay-accelerating factor in coxsackievirus A21-mediated cell infectivity**. *J Virol* **78**:12677-12682.
14. Au GG, Lindberg AM, Barry RD, and Shafren DR (2005) **Oncolysis of vascular malignant human melanoma tumors by Coxsackievirus A21**. *Int J Oncol* **26**:1471-1476.
15. Shafren DR, Au GG, Nguyen T, Newcombe NG, et al. (2004) **Systemic therapy of malignant human melanoma tumors by a common cold-producing enterovirus, coxsackievirus a21**. *Clin Cancer Res* **10**:53-60.
16. Au GG, Lincz LF, Enno A, and Shafren DR (2007) **Oncolytic Coxsackievirus A21 as a novel therapy for multiple myeloma**. *Br J Haematol* **137**:133-141.

17. Regidor PA, Callies R, Regidor M, and Schindler AE (1998) **Expression of the cell adhesion molecules ICAM-1 and VCAM-1 in the cytosol of breast cancer tissue, benign breast tissue and corresponding sera.** Eur J Gynaecol Oncol **19**:377-383.
18. Rosette C, Roth RB, Oeth P, Braun A, et al. (2005) **Role of ICAM1 in invasion of human breast cancer cells.** Carcinogenesis **26**:943-950.
19. Madjd Z, Durrant LG, Bradley R, Spendlove I, et al. (2004) **Loss of CD55 is associated with aggressive breast tumors.** Clin Cancer Res **10**:2797-2803.
20. Lublin DM and Atkinson JP (1989) **Decay-accelerating factor: biochemistry, molecular biology, and function.** Annu Rev Immunol **7**:35-58.
21. Shafren DR, Bates RC, Agrez MV, Herd RL, et al. (1995) **Coxsackieviruses B1, B3, and B5 use decay accelerating factor as a receptor for cell attachment.** J Virol **69**:3873-3877.
22. Coyne KE, Hall SE, Thompson S, Arce MA, et al. (1992) **Mapping of epitopes, glycosylation sites, and complement regulatory domains in human decay accelerating factor.** J Immunol **149**:2906-2913.
23. Boyd AW, Wawryk SO, Burns GF, and Fecondo JV (1988) **Intercellular adhesion molecule 1 (ICAM-1) has a central role in cell-cell contact-mediated immune mechanisms.** Proc Natl Acad Sci U S A **85**:3095-3099.
24. Dougherty RM (1964) Animal virus titration techniques. In: Harris RJC (ed) Techniques in Experimental Virology, Academic Press, New York
25. Mullen JT and Tanabe KK (2002) **Viral oncolysis.** Oncologist **7**:106-119.
26. Shafren DR (1998) **Viral cell entry induced by cross-linked decay-accelerating factor.** J Virol **72**:9407-9412.
27. Stuart AD, Eustace HE, McKee TA, and Brown TD (2002) **A novel cell entry pathway for a DAF-using human enterovirus is dependent on lipid rafts.** J Virol **76**:9307-9322.
28. Kunz-Schughart LA, Kreutz M, and Knuechel R (1998) **Multicellular spheroids: a three-dimensional in vitro culture system to study tumour biology.** Int J Exp Pathol **79**:1-23.
29. Carrick S, Gherzi D, Wilcken N, and Simes J (2004) **Platinum containing regimens for metastatic breast cancer.** Cochrane Database Syst Rev CD003374.
30. Sampath D, Discafani CM, Loganzo F, Beyer C, et al. (2003) **MAC-321, a novel taxane with greater efficacy than paclitaxel and docetaxel in vitro and in vivo.** Mol Cancer Ther **2**:873-884.
31. Sulkin SE, Schwab M, and Wallis HC (1951) **Isolation of coxsackie viruses; litter differences among suckling mice.** Proc Soc Exp Biol Med **77**:354 - 356.
32. Ciugarin-Brailoiu M (1975) **Variations in Coxsackie virus pathogenicity in the course of routine isolations in suckling mice and cell cultures.** Virologie **26**:81 - 86.
33. Mufson MA, Kawana R, Bloom HH, Gorstein F, et al. (1968) **Pathogenicity of Coxsackie A-21 virus for suckling mice.** Proc Soc Exp Biol Med **128**:237-246.
34. Fisher K (2006) **Striking out at disseminated metastases: the systemic delivery of oncolytic viruses.** Curr Opin Mol Ther **8**:301-313.

35. Fu X and Zhang X (2002) **Potent systemic antitumor activity from an oncolytic herpes simplex virus of syncytial phenotype.** *Cancer Res* **62**:2306-2312.
36. Zhang JF, Hu C, Geng Y, Selm J, et al. (1996) **Treatment of a human breast cancer xenograft with an adenovirus vector containing an interferon gene results in rapid regression due to viral oncolysis and gene therapy.** *Proc Natl Acad Sci U S A* **93**:4513-4518.
37. Norman KL, Coffey MC, Hirasawa K, Demetrick DJ, et al. (2002) **Reovirus oncolysis of human breast cancer.** *Hum Gene Ther* **13**:641-652.
38. Fernandez M, Porosnicu M, Markovic D, and Barber GN (2002) **Genetically engineered vesicular stomatitis virus in gene therapy: application for treatment of malignant disease.** *J Virol* **76**:895-904.
39. Magee WE and Miller OV (1970) **Individual variability in antibody response of human volunteers to infection of the upper respiratory tract by coxsackie A21 virus.** *J Infect Dis* **122**:127-138.
40. Patel N, Buthala DA, and Walker JS (1964) **Controlled Studies of Coxsackie a-21 (Coe) Virus in Volunteers.** *J Infect Dis* **114**:87-94.
41. Pereira MS and Pereira HG (1959) **Coe virus. Properties and prevalence in Great Britain.** *Lancet* **2**:539-541.
42. Nwanegbo E, Vardas E, Gao W, Whittle H, et al. (2004) **Prevalence of neutralizing antibodies to adenoviral serotypes 5 and 35 in the adult populations of The Gambia, South Africa, and the United States.** *Clin Diagn Lab Immunol* **11**:351-357.
43. Xu F, Sternberg MR, Kottiri BJ, McQuillan GM, et al. (2006) **Trends in herpes simplex virus type 1 and type 2 seroprevalence in the United States.** *JAMA* **296**:964-973.
44. Selb B and Weber B (1994) **A study of human reovirus IgG and IgA antibodies by ELISA and western blot.** *J Virol Methods* **47**:15-25.
45. Tai JH, Williams JV, Edwards KM, Wright PF, et al. (2005) **Prevalence of reovirus-specific antibodies in young children in Nashville, Tennessee.** *J Infect Dis* **191**:1221-1224.