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A Novel Approach for Detecting Viable and Tissue-specific Circulating Tumor Cells through an Adenovirus-based Reporter Vector

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Abstract

BACKGROUND—Circulating tumor cells (CTCs) hold great promise as biomarkers and are a direct source of tumor cells through a simple blood draw. However, CTCs are rare and their detection requires sensitive and specific methods to overcome the overwhelming hematocyte population. Therefore, CTC detection remains technically challenging.

METHODS—An assay was developed for detecting viable and tissue-specific CTCs using a tropism-enhanced and conditionally-replicating reporter adenovirus (CTC-RV). Adenoviral replication was made prostate-specific by placing the E1A gene under the control of the probasin promoter and prostate specific antigen enhancer (PSE-PBN). Viral tropism was expanded through capsid-displayed integrin targeting peptides. A secreted reporter, humanized *Metridia* Luciferase (hMLuc), was engineered for expression during the major late phase of viral replication. The assay involves red blood cell lysis, cell collection, viral infection and subsequent quantification of reporter activity from cellular media. Assay and reporter stability, cell specificity and sensitivity were evaluated in cell dilution models in human blood.

RESULTS—A conditionally replicating prostate-selective adenovirus reporter and CTC assay system were generated. The secreted reporter, MLuc, was found to be stable for at least three days under assay conditions. CTC detection, modeled by cell dilution in blood, was selective for androgen receptor positive prostate cancer cells. Serial dilution demonstrated assay linearity and

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SUPPLEMENTAL INFORMATION Supplementary table 1. Applied primers and linkers.

sensitivity to as few as 3 cells. Prostate cancer cell viability declined after several hours in anticoagulated blood at ambient temperatures.

CONCLUSIONS—Conditionally replicative adenoviral vectors and secreted reporters offer a functional method to detect viable CTCs with cell specificity and high sensitivity.

Keywords

Prostate Cancer; Circulating Tumor Cells (CTCs); CTC Reporter Virus (CTC-RV); Metridia Luciferase; Adenovirus; Coxsackie-Adenovirus Receptor (CAR); RGD Peptide

INTRODUCTION

Tumor cell metastasis is the major cause of cancer-related death. This process is initiated by migratory cancer cells that escape the local tumor environment and travel to distant sites through adjacent tissue, nervous, lymphatic, or circulatory systems. In addition to active metastasis, tumor cells may be passively shed during tissue remodeling and tumor growth [1]. This escape of tumor cells from primary and metastatic lesions generates a population of cells known as disseminated tumor cells (DTCs). A portion of these cells become partitioned into the circulatory system and are thus known as CTCs. Evidence of CTCs has been present for over a century [2]; however their capture and study has been limited until recently. Several developing CTC detection technologies, including one FDA approved platform, have shed light on the potential value of CTCs in patients with prostate cancer (PCa).

The clinical value of CTCs in PCa was first recognized in patients with metastatic disease, where the number of CTCs per milliliter of blood was found to be indicative of survival and therapeutic response [3–6]. In addition to CTC enumeration, new and detailed studies of CTC biology such as genetics and gene expression are indicating that CTCs may be a future source for personalized cancer treatments [7–14]. Moreover, the increasing ability to detect DTCs in patients with non-metastatic disease suggests that CTC technology may soon benefit men with localized PCa [10,15–17]. There has therefore been great interest and effort to develop new and improved techniques for the identification and characterization of CTCs.

CTC detection technologies have principally consisted of two strategies: (i) the capture and/or labeling of cells by immunologic methods and (ii) the genetic analysis of mixed cell populations. These approaches generally begin with a step to enrich tumor cells from the blood, such as density gradient centrifugation, immunomagnetic capture of epithelial cells, immunomagnetic depletion of mononuclear cells or size filtration [18–25]. Tumor cell enrichment is critical because CTCs are very rare and are estimated at only one per billion normal blood cells [26]. After enrichment, the remaining population is often still predominantly White Blood Cells (WBCs) and therefore additional processing is required to detect or distinguish CTCs from remaining leukocytes. Direct CTC analyses, which are capable of studying individual cells, include immunologic staining, flow cytometry and *in situ* hybridization [7,10,27–29]. Indirect genetic analyses for CTC detection include RT-PCR, sequencing and comparative genomic hybridization for known cancer-associated markers [9,11,12,30,31]. Cutting edge technologies have made it possible to isolate

individual cells for molecular analyses such as gene expression microarrays, sequencing and RT-PCR [13,14,32]. Despite these great advances in technology, there are still many challenges in CTC detection and characterization. These include the ability to determine CTC viability and to perform direct biologic assays on viable CTCs. One developing approach to address these limitations involves recombinant viral vectors [33].

Recombinant adenoviruses have been the most broadly applied viral vector in experimental therapeutics. The first tissue-specific oncolytic cancer gene therapy was developed for prostate cancer in the 1990's [34], and applied a Conditionally Replicative Adenovirus (CRAd). Prostate specificity was achieved through the use of the Prostate Specific Antigen (PSA) promoter and enhancer, which induced early viral gene expression, viral replication and cell lysis. The resulting viruses were translated to multiple clinical trials for the treatment of local and metastatic PCa [35–37]. Following these initial efforts, there have been several new advances in adenoviral vector technology. These include capsid modification for targeted viral infection [38–40], Major Late Transcriptional Unit (MLTU) expression cassettes for specific and high level transgene expression [41–43], and genetic modifications for enhanced viral replication rates [44]. While all of these advances were developed for gene therapy, they are equally applicable or translatable to viral-based CTC detection technology. Here we apply tissue-specific CRAd technologies for the development and characterization of a prostate-specific reporter virus. This viral reporter can detect small numbers of prostate cancer cells in the background of the abundant WBCs in peripheral blood. We believe that this approach offers a flexible and inexpensive platform that could be applied for CTC detection of most cancers.

MATERIALS AND METHODS

Cell lines

LNCaP, 786-0, ACHN and HT1376 cells were obtained from ATCC (Manassas, VA) and were maintained as recommended. C4-2 cells were obtained from Johns Isaacs (Johns Hopkins University, Baltimore, MD) and maintained in RPMI-1640. DPL-S11 cells were maintained in DMEM as previously described [44]. LMD cells are a derivative of MDA-MB-231 cells and were provided by Sridhar Nimmagadda (Johns Hopkins University, Baltimore, MD). LNCaP-MLuc are stable LNCaP cells derived to express the hMLuc reporter through the h β -Actin promoter and enhancer; these cells are maintained under blasticidin selection as previously described [45]. All media contained 10 μ g/mL ciprofloxacin hydrochloride (U.S. Biological, Swampscott, MA) and 10% fetal bovine serum.

Plasmid construction

A Fiber gene shuttle vector containing an integrin targeted Fiber protein and the hMLuc reporter gene was constructed in a stepwise manner. First, a recombinant fragment including Fiber, followed by an Internal Ribosome Entry Site (IRES) and hMLuc was generated. The coding region of hMLuc was amplified by PCR with primers hMLuc-Fiber5/hMLuc-Fiber3 (Supplementary Table 1) from pDonor-hB-Actin-hMLuc [45]. The resulting PCR product was subcloned into the BamHI/NotI site of RpucFBR1-IRES-HSVTK, replacing HSVTK,

generating R_{puc}-FBR1-IRES-hMLuc. The coding sequence of the integrin binding peptide, RGD4C, was constructed by annealing oligos RGD4C 5/RGD4C 3 (Supplementary Table 1) and the resulting linker was subcloned into the BspE I site of R_{puc}-FBR1-IRES-hMLuc, generating R_{puc}-FBR3-IRES-hMLuc, which contained an intact CAR binding site and an integrin binding site. Six His repeats were then added to the 5'-end of MLuc through PCR with primers MLucSense/MLucAntisense (Supplementary Table 1). The resulting product was subcloned into the BamHI/NotI site of R_{puc}-FBR3-IRES-MLuc, generating R_{puc}-FBR3-IRES-histagged-hMLuc vector. Finally, a 576 bp fragment from R_{puc}-FBR1-p21-shRNA, containing a p21 shRNA expression cassette, was subcloned via NotI [44], generating fiber shuttle vector R_{puc}-FBR3-IRES-histagged-hMLuc-p21shRNA. All shuttle vectors were verified and confirmed by restricted-enzyme digestions and sequencing with primers SeqMLuc, SeqHisTag-hMLuc, and SeqRGD4C (Supplementary Table 1). Primers were obtained from Sigma (St. Louis, MO) and all restriction enzymes were purchased from New England BioLabs (Ipswich, MA).

Generation of recombinant adenovirus

The fiber shuttle vector R_{puc}-FBR3-IRES-histagged-hMLuc-p21shRNA was recombined into the Fiber region of pseudotyped Ad5 adenovirus, Ad5-PSE-PBN-E1A-Fex, by Cre mediated cassette exchange as previously described [40,46]. Briefly, C4-2 cells were plated in a T75 flask one day before transfection. The next day cells were co-transfected with R_{puc}-FBR3-IRES-histagged-hMLuc-p21shRNA and R_{puc}-Cre plasmid (1μg/ml) using Magnet Assisted Transfection (IBA BioTAGnology, St. Louis, MO) in cellular media containing 5 nM R1881. The next day cells were infected with Ad5-PSE-PBN-E1A-Fex (MOI=1). Recombinant virus was harvested 72 hours later and amplified in DPL-S11 cells, which are a derivative of DPL cells and expresses the fiber specific single chain antibody S11 [44,47]. The amplified virus was purified with the Adenopure™ (Puresyn, PA) and aliquots were stored at -80°C. The virus titer was determined by Adeno-XTM Rapid Titer Kit (BD Biosciences, San Jose, CA).

CTC-RV assays

Cells were spiked into one ml blood of human blood from discarded and pooled male clinical samples that had been collected and stored in K₂ EDTA vacutainer tubes at 4 °C for 4–8 weeks. The resulting blood and cell mixture was treated with RBC lysis buffer (Roche Diagnostics, Indianapolis, IN) and incubated at 37°C for 15 min. After centrifugation, the cell pellets were re-suspended and washed using PBS to remove serum protein and other components. The cell pellets were then suspended and infected with 2×10³ to 1×10⁶ Plaque Forming Units (PFU) of CTC-RV in three milliliters of cell culture media containing 5 nM R1881. 2×10⁵ PFU of CTCRV was applied for specificity and sensitivity assays. After four hours of infection, the virus was removed and cells fed with three milliliters of fresh complete cell culture media containing 5 nM R1881. The samples were maintained at 37°C in an atmosphere containing 5% CO₂ and cellular supernatant was harvested three days post infection. Secreted His-Tagged hMLuc was captured by resin using TALON metal affinity resin according to manufacturer's protocol (Clontech, Mountain View, CA). For each sample twenty-five μl of washed MLuc-resin was placed into individual wells of a 96 well solid white flat bottom polystyrene plates (Corning, Lowell, MA) and assayed for hMLuc activity

using 100 μ l of assay buffer (PBS, pH= 7.0 containing 2.86 μ M coelenterazine) (Biosynth AG, Swaziland). Assays were read in a Perkin Elmer Micro Beta luminometer (Perkin Elmer, Shelton, CT).

Cell detection limit, cell viability and cell type specificity studies

For cell detection limit studies, LNCaP cells (0–100) were spiked into one ml blood and applied to the CTC-RV assay. For cell type specificity studies LNCaP, C4-2, 786-0, LMD, ACHN and HT1376 cells (1000 cells) were spiked into one ml of blood and applied to the CTC-RV assay. Samples were processed through the CTC-RV assay as described above. For cell viability studies samples were assayed after incubation at room temperature for 0–24 hours.

hMLuc protein stability study

One thousand LNCaP cells were spiked into human blood from discarded male clinical samples. The resulting blood and cell mixture was processed by RBC lysis and washed with PBS as described above. The cell pellets were then suspended using complete cell culture media and a separate source of hMLuc, from LNCaP-MLuc conditioned media, was then added to the cells and media mixture. The samples were kept at 37°C in an atmosphere containing 5% CO₂. Aliquots of cellular media were sampled at various time points (0, 1, 3, 6 and 9 days) and processed MLuc activity assay as described above.

Statistical analysis

Statistical evaluations were performed by t-test. A p-value of $P < 0.05$ was considered as statistically significant and $P < 0.0001$ as highly statistically significant. Linear regression analysis was performed for serial dilution studies.

RESULTS

CTC-RV Vector Concept and Assay Design

There is considerable rationale for developing tissue-specific conditionally replicative reporter adenovirus for CTC detection. The human adenovirus serotype 5 (Ad5) primarily infects epithelial cells, but not WBCs [48], and thus naturally overcomes a major background problem in CTC detection. Adenovirus reporters also only detect viable cells, adding a potentially important parameter to CTC detection and quantification. Late viral gene regions can also be utilized for reporter gene expression, which will limit gene expression until after the adenoviral genome has been amplified [49,50]. This genomic replication provides an intrinsic mechanism for reporter gene amplification of up to 10,000 copies [51]. Finally, the application of secreted reporters can separate the CTC signal from the overwhelming background of WBCs and cell debris.

The schematic in Figure 1 summarizes the primary design of the CTC-RV constructs and assay. The two essential functional units are the conditional replication cassette, in the immediate early gene region, and the secreted reporter cassette within the Major Late Transcriptional Unit (MLTU) (Fig. 1, top panel). Selective or conditional viral replication can be achieved by replacing the endogenous E1A promoter with a tissue-specific or

pathway specific gene promoter [52]. Reporter gene expression in the late phase of the viral lifecycle can be achieved by transcriptionally linking the reporter to capsid genes, such as Fiber, through an internal ribosomal entry site (IRES). With this approach the viral replication and reporter gene expression are made tissue or pathway specific.

The CTC-RV assay first isolates a large population of cells from the peripheral blood by RBC lysis and centrifugation (Fig. 1, lower panel). This cell population consists of WBCs, CTCs, and other disseminated cells and debris. The cell pellets are briefly washed and then infected with the CTC-RV vector. While the recombinant virus can theoretically infect most any epithelial cell in this population, it is designed to selectively replicate and activate reporter expression in cells with the active or tissue specific pathway. Cellular supernatant is then harvested and CTC-RV activity is measured by secreted reporter activity. The resulting vector design allows for the replacement of the viral replication cassette or the reporter cassette if alternate promoters or reporters are desired. Thus, one can readily generate CTC-RVs for alternate diseases, reporters or pathways.

Prostate-selective CTC-RV

The serotype 5 adenoviral vector system, pFex [46], was applied to generate a prostate-selective CTC-RV with modified E1 and Fiber gene regions. Prostate-selective viral replication was engineered through the use of the probasin (PBN) promoter and the PSA enhancer (PSE) to initiate E1A gene expression (Fig. 2). The use of these prostate specific promoters and enhancers for conditional adenoviral replication is well established in the gene therapy field [34,40,53–56]. A polyhistidine-tagged and secreted reporter gene, His-hMLuc, was then transcriptionally linked to the Fiber gene through an IRES. This approach has previously been applied to temporally regulate reporter gene expression until after the viral genome has replicated [43,57,58]. In addition to these features, viral tropism was expanded by inclusion of the integrin targeting peptide, RGD4C, into the Fiber HI-loop region as previously described [46]. Therefore the virus has been designed to infect CTCs through the natural Coxsackie and Adenovirus Receptor (CAR) as well as through epithelial integrins [59]. Finally, a shRNA expression cassette targeting p21/ WAF1 was incorporated downstream of Fiber to amplify the rate of viral replication [44]. The resulting CTC-RV was amplified and packaged in DPL-S11 cells, a derivative of PER.C6 which is designed to eliminate the development of wild-type E1 Replication Competent Adenovirus [44,47]. The resulting virus was quantified by standard plaque forming assays.

The stability of the hMLuc enzyme in CTC-RV assay conditions

The replication of recombinant Ad5 viruses can require 24–96 hours for completion. Therefore, we sought to determine the stability of the hMLuc reporter in CTC-RV assay conditions over several days. To simulate CTC-RV assay conditions 1,000 LNCaP cells were spiked into human blood. The blood was then processed by RBC lysis and centrifugation. The resulting cell pellet was then suspended in complete cell culture media (as summarized in steps 1–2 of Fig. 1, lower panel). An exogenous source of hMLuc protein, from LNCaP-MLuc conditioned media [45], was then added and the samples were incubated at 37°C in an atmosphere containing 5% CO₂ for up to 9 days. No viruses were added for this experiment. Aliquots were sampled at specified times and analyzed for MLuc

enzymatic activity. The relative MLuc activity is reported relative to day 0, which was sampled immediately following the addition of MLuc (Fig. 3). There was no significant loss of MLuc activity for up to three days. However, the level of activity was observed to be mildly lost at days six and nine. These results support that the MLuc reporter protein can be stably evaluated for up to three days in the CTC-RV assay without significant degradation.

CTC-RV infection conditions

Infection conditions were investigated to determine the optimal viral dose for infection and reporter gene expression. As a model, fifty LNCaP cells were spiked per milliliter of human whole blood, and the blood was processed by RBC lysis and centrifugation. The resulting cellular pellets were then suspended in culture media and infected with various doses of the prostate CTC-RV ranging from 2×10^3 to 1×10^6 Plaque Forming Units (PFU). After four hours the virus was removed, cells were washed, and the cell pellet suspended in standard culture media. The infected cells were incubated for three days at $37^\circ\text{C} / 5\% \text{CO}_2$. On the third day the samples were harvested and the supernatant isolated following centrifugation of the cell pellet. The secreted His-tagged hMLuc was captured from the media by cobalt resin, was suspended in PBS and then assayed for MLuc Activity. The results indicate 2×10^5 PFU to be the most effective dose for detecting CTCs in this model (Fig. 4). Higher viral doses resulted in reduced activity, possibly due to viral induced toxicity. For all future described experiments a dose of 2×10^5 PFU of prostate CTC-RV was applied.

Prostate CTC-RV assay cell detection limit and linearity

The cell detection limit and linearity of the CTC-RV assay was determined by diluting a range of LNCaP cells to a concentration of 1–100 cells per ml of human blood. Negative control samples, without LNCaP cells (No Cell Control), were included for background subtraction. Following RBC lysis, the samples were centrifuged, washed and infected with 2×10^5 infectious units of the prostate-specific CTC-RV. After four hours the virus was removed and the cells were plated in complete cell culture media and grown under standard cell culture conditions. Three days later the resulting secreted MLuc activity was measured from the cellular supernatant. The resulting CTC-RV signal Units are presented as Counts Per Second (CPS) of MLuc luminescent activity after subtraction of control blood without LNCaP cells (No Cell Control). The signals were highly linear over these five dilutions ($R^2 = 0.9788$), which represent a clinically relevant range of CTC density (Fig. 5). A minimum detection limit of three cells was readily achieved with the assay.

The Cell Type Specificity of the Prostate CTC-RV Assay

The probasin promoter and PSE enhancer are responsive to the Androgen Receptor (AR) steroid nuclear transcription factor, which is active in the majority of advanced human prostate cancers, including castration resistant prostate cancer [60]. To evaluate the specificity of these components in the CTC-RVs assay, we separately spiked human blood with either AR-positive prostate cancer cells: LNCaP or C4-2, or AR negative control cell lines from renal cancer: 786-0, ACHN and HT1367 or breast cancer: LMD-MDA-MB-231. The signal detected from AR-positive prostate cancer cell lines was significantly higher than those of non-prostate cells (Fig. 6). These results reflect previous studies of prostate CRAD selectivity. Some mild background signal was detectable in non-prostatic cell lines. This

minimal background may represent leakiness of the promoter, alternate mechanisms of adenoviral replication through defective p53 or Rb pathways, or complementation from endogenous viral genes.

Stability of prostate cancer cell viability in blood

CTC viability and detection may be influenced by the length of time between blood draw and CTC-RV assay. We sought to determine the window of time that CTCs, represented by LNCaP or C4-2 cells, could remain viable in blood at room temperature and still be detected by the CTC-RV assay. 1,000 LNCaP or C4-2 cells were spiked into one ml of human blood and mixed gently. Samples were then kept at room temperature (22–25 °C) for various lengths of time (0, 3, 6, 12, and 24 h). Each sample was then processed in parallel by the CTC-RV assay as described above. Over time, the relative CTC-RV signal degraded, indicating a cumulative loss of prostate cancer cell viability over the 24 hour time period. Nonetheless, the cells still remained detectable after 24 hours at room temperature when compared to control blood without cancer cells (Fig. 7). An optimal time for viable CTC detection may therefore be within the first several hours after blood draw, where a smaller fraction of signal is lost. The loss of signal may be reduced or overcome if rapid processing protocols are established or if blood storage conditions are developed to retain or improve CTC viability.

DISCUSSION

The ability to capture and characterize CTCs has improved enormously over the last decade. Next generation CTC technologies are becoming more efficient at cell enrichment by exploiting differences between CTCs and WBCs. New methodologies that are not dependent upon EpCAM expression increase the potential to detect diverse CTC populations such as EMT-like cells or other poorly differentiated CTCs [13]. Serotype 5 adenoviral CTC reporters utilize the viral receptor, CAR, as an alternate cell surface epithelial ligand for CTC detection[33]. Our results expand upon this by developing a tropism modified Ad5 viruses which can infect CTCs through CAR as well as through epithelial integrins. These modifications may be beneficial for detecting CTCs which express lower levels of EpCAM or CAR [61].

One attribute for Ad5 reporters in CTC analysis is their natural inability to infect WBCs. This resistance is primarily due to the absence of CAR expression [48]. However, studies with a telomerase-based CTC adenovirus reporter indicate that WBCs can be infected when very high doses of infectious virus are applied [62]. To avoid potential WBC infection by this mechanism we applied CTC-RV doses that are lower than those reported with the telomerase vector. While WBCs may still be infected under these conditions, we have not observed significant signal from controls samples lacking PCa cells. The results suggest that the prostate CTC-RV assay produces negligible signal from WBCs. This may be due to a combination of inefficient WBC infection and tissue-selective activity of the PSE-PBN promoter and enhancer. It is notable that low level CTC-RV signal was detectable in non-prostatic cells. It is not clear whether this signal is from leaky expression of the PSE-PBN promoter and enhancer or if weak viral replication is occurring through cancer-associated

pathways. We believe that these non-specific signals are minimal and will not significantly impact the assay in cancer-free individuals because normal epithelial cells will not be viable due to anoikis [63]. One exciting aspect of CTC-RVs is the potential to study specific signaling pathways *in situ* in living cancer cells. Telomerase promoter driven adenovirus reporters have successfully detected viable CTC from patients with gastric, breast, colon, and gynecological cancers [33,62,64,65]. In this study we selected the AR signaling pathway due to its selectivity and importance in prostate cancer. AR mutations, AR-splice variants and AR gene amplification have been reported in CTCs from patients with castration resistant prostate cancer [9,29]. These mutations are likely associated with aberrant AR activity. More recently, AR activity has been studied in captured CTCs where PSA and PSMA immunofluorescent staining served as surrogate marker for AR signaling activity [6]. These results by Miyamoto and colleagues indicate that measurement of AR signaling levels in CTCs before and after androgen ablative therapies may be predictive of adverse therapeutic outcomes. We anticipate that *in situ* analysis of AR activity in viable CTCs through the CTC-RVs could also prove useful in predicting response to androgen ablative therapies.

One distinction of the CTC-RV approach described here is the application of a secreted reporter. Secreted reporters may offer an advantage because they separate the CTC signal from the overwhelming population of hematologic cells. The secreted MLuc bioluminescent reporter is highly sensitive and has been previously used to linearly measure viable cell numbers over several logs of dilution [45]. In this study we have further reported the stability of MLuc in tissue culture medium over several days in culture. While secreted reporters can have these advantages, the volume of media required to culture CTCs and peripheral blood cells can be large. For example, here we have applied 3 milliliters of culture media for each milliliter of blood. To address the potential for large volume cultures, we utilized His-tagged MLuc and affinity-based resin capture. These modifications increased the number of steps in the assay, but significantly increased the signal intensity. The result is a highly sensitive CTC assay, to as few as 3 cells, which is comparable with the sensitivity and recovery rates other modern CTC detection assays [16,64].

Another notable distinction of the CTC-RV approach is the nature of the result, which is a bioluminescent reporter signal rather than cellular enumeration. The level of CTC-RV signal will be influenced by a combination of many factors beyond CTC number, such as the extent of pathway activity, CTC infection capacity, cellular fitness, cellular viability and tumor cell heterogeneity. Clinical studies will be required to determine what signal ranges are associated with an unfavorable clinical outcome and whether or not the quantity of the signal has a correlative relationship with disease severity or response to therapy. Because this is a biologic assay, there is also potential for assay variability based on day to day assay conditions. This may be accounted for by including a reference control, such as purified hMLuc or a stable PCa cell line, to normalize for slight changes in assay conditions.

A final aspect to be considered in reporter-based CTC detection assays is the time period between blood draw and assay. Isolated CTCs face many stresses including exposure to altered oxygen densities and temperatures in sample tubes, to physical stress during partitioning and transfer, and finally by exposure to a new substratum and environment in

cell culture. The combination of these stresses, tumor cell heterogeneity, and the unknown optimum conditions for CTC cell culture has made the assessment of CTC viability challenging. This is particularly challenging for prostate cancer, where it has been rare to develop long term growth of adenocarcinoma cell lines. The consequences of CTC processing are evident in Figure 7, where approximately half of the spiked cells died within the first four hours of incubation in blood. Therefore CTC assays which utilize viable cell activity, or which measure CTC viability, should be done as quickly as possible after blood draw. The potential for cell death or inadequate growth conditions should also be considered when interpreting results. New techniques may be developed to overcome some of these limitations in future CTC assays.

CONCLUSION

In conclusion we have developed and characterized a novel prostate cancer CTC detection assay, based on tissue-specific promoter driven conditionally replicative serotype 5 adenovirus and secreted bioluminescent reporters. The assay is highly sensitive and selective for prostate cancer cells. In future developments of the assay we aim to streamline the process, to include reference controls for normalization and to improve the viability and lifespan of CTCs following blood draw. This approach may offer new insights into CTC biology and cancer prognosis.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1. CTC reporter vector concept and assay Design

(Top panel) Selective adenoviral replication is achieved by replacing the early viral E1A gene promoter with a tissue-specific or pathway specific promoter in the immediate early gene region. Following viral genomic replication, the MLTU is activated. The secreted reporter is then expressed as part of the MLTU through linkage to the Fiber capsid gene with an IRES. (Lower panel) The CTC-RV assay utilizes anticoagulated whole blood which is first treated with RBC lysis buffer and centrifugation. The resulting cell pellet is then infected with CTC-RV and maintained under standard cell culture conditions. After three days the secreted reporter activity is measured in the supernatant, separating the CTC signal from the large population of cells. MLP; Major Late Promoter

Fig. 2. Vector map of the prostate CTC-RV

The viral vector is a serotype 5 adenovirus derived from pAdEasy-1 and pFex. The vector is E3 deleted. The E1 region contains the PSA Enhancer and Probasin Promoter which drive E1 gene expression and viral replication. The Fiber gene cassette was inserted through pFex Cre/Lox recombination and therefore contains two flanking Lox sites. The Fiber protein is modified for integrin targeting through HI-loop display of the RGD4C peptide. The CAR-binding sites of fiber are intact. An IRES is downstream of Fiber and it mediates translation of His-tagged hMLuc. Downstream of the polyadenylation signal is a p21 shRNA, driven by the H1 promoter, to accelerate viral replication.

Fig. 3. Stability of hMLuc protein in CTC-RV assay

To simulate CTC-RV assay conditions 1,000 LNCaP cells were spiked into human blood. The blood was then processed by RBC lysis and centrifugation and the resulting cell pellet was suspended in complete cell culture media. Exogenous hMLuc was added and samples were incubated under standard cell culture conditions. Aliquots were sampled at specified times and analyzed for MLuc enzymatic activity assay. The relative MLuc activity is reported relative to day 0. Asterisks indicate statistically significant degradation (unpaired t-test). Error bars represent standard error of the mean of at least three replicates. Activity loss begins at approximately 6 days in culture. *, $P < 0.05$.

Fig. 4. Infection conditions for CTC-RV assay

50 LNCaP cells were diluted into one ml of human blood and processed by RBC lysis. The cell pellets were infected with increasing doses (PFU, Plaque Forming Units) of the prostate CTC-RV. Three days after infection the media were collected and assessed for MLuc activity. CTC-RV signal Units are presented at Counts per Second (CPS) of MLuc luminescent activity after subtraction of background signals (each CTC-RV dose with no cell control). Error bars represent standard error of the mean of at least three replicates.

Fig. 5. High sensitivity detection of prostate cancer cells in blood

1–100 LNCaP cells were spiked into one ml blood and processed by the CTC-RV assay.

CTC-RV signal Units are presented at Counts per Second (CPS) of MLuc luminescent

activity per sample after subtraction of control blood without LNCaP cells (No cell control).

Error bars represent standard error of three independent assays. Linear regression analysis:

$R^2 = 0.9788$.

Fig. 6. The cell type specificity of the prostate CTC-RV assay

1,000 LNCaP, C4-2, 786-0, LMD-MDA-MB-231 (LMD), ACHN and HT1376 cells were separately spiked into one ml of human blood. All samples were then processed in parallel for the prostate CTC-RV assay. CTCRV signal Units are presented at Counts per Second (CPS) of MLuc luminescent activity after subtraction of control blood without cancer cells (No cell control). Error bars represent standard error of the mean of three replicates. Statistical evaluation: ***, $P < 0.0001$, indicates statistically highly significant differences relative to each non-prostate cell lines (t-test).

Fig. 7. Stability of viable prostate cancer cells in blood

LNCaP and C4-2 cells (1000 cells) were spiked into one ml of human blood and incubated at room temperature for 0, 3, 6, 12, and 24 hours. All samples were then processed in parallel for the prostate CTC-RV assay. CTC-RV signal Units are presented at Counts per Second (CPS) of MLuc luminescent activity after subtraction of control blood without cancer cells (No cell control). Error bars represent standard error of the mean of at least three replicates.