Gamma-delta T Cells may Function as Carrier Vehicles in Adenovirus Vector-based Gene Therapy

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Abstract

Developing new treatments that reduce prostate cancer progression is important. Therapeutic efficacy of conventional gene therapy for metastatic prostate cancer is still low. Lower induction rate of naked genes into target cell is due to reduced expression of adenovirus receptor on cancer cell and also due to high seroprevalence of anti-Ad antibodies in adults. Therefore, efficient Ad carrier systems that circumvent these problems should be developed. Gamma-delta T cells have demonstrated high affinity to cancer cells. CD46, which leads to broad tropism in Ad35 vectors, is expressed in hematopoietic cells, including $\gamma\delta$ T cells. In this study, we demonstrate the potential of $\gamma\delta$ T cells as "vehicles" for transporting Ad5/F35 vectors and genes into cancer cells.

Keywords: Prostate cancer; Gamma-delta T cell; Adenovirus vector; Adenovirus serotype; Gene transduction efficacy

Introduction

Although conventional therapies, such as surgery and/or radiation, cure the majority of patients with localized prostate cancer, some patients will relapse and a substantial fraction will die. Although androgen deprivation therapy (ADT) is the principal treatment for these patients, it is not curative and typically fails. After patients with prostate cancer become ADT-refractory, chemotherapeutic reagents, such as taxane, are often the next line of treatment. Although chemotherapy has proven survival benefits, many patients frequently relapse. Identification of new therapies that reduce prostate cancer progression would be a remarkable breakthrough in treatment. Gene therapy and immunotherapy are considered fourth or fifth lines of treatment for prostate cancer. Many adenovirus (Ad) vector-based gene therapies have been investigated for metastatic prostate cancer in recent years. However, the therapeutic efficacy of Ad vector-based gene therapy is lower than expected due to different characteristics of various Ad vector serotypes.

A total of 51 different human Ad serotypes have been isolated and are classified into six distinct subgroups [1]. Ad type 5 (Ad5), which belongs to subgroup C, is commonly used to prepare recombinant Ad vectors due to extensive knowledge regarding its genetic and biological characteristics. However, Ad5-based gene therapy has issues that need to be addressed. Ad5 requires coxsackievirus and adenovirus receptors (CARs) on the cell membrane of target cells for infection [2,3]. Interaction between the Ad5 fiber knob and CARs is a key mediator through which Ad infects target cells. However, CAR expression in cancer cells is often down-regulated, which is an obstacle in Ad5 infection of target cells (Figure 1). To overcome low Ad5 infection rates in cells lacking CARs, Mizuguchi and Hayakawa developed a novel Ad5/F35 vector with chimeric type 5 and type 35 fiber proteins [4,5]. Ad5/F35 is capable of CAR-independent tropism in target cells that express CD46 on the membrane. CD46 was reported to be a membrane cofactor protein and receptor for subgroup B Ads (serotypes 3, 7, 11, 14, 16, 21, 34, 35, 50 and 51 [6,7]). Human CD46 is expressed in the majority of human cells, including hematopoietic cells, leading

to broad tropism of Ad35 vectors. Because Ad35 has a high affinity for hematopoietic cells [8], we hypothesized that $\gamma\delta$ T cells could be candidate vehicles for Ad5/F35 delivery to target cells. $\gamma\delta$ T cells are a small subset (1-10%) of peripheral blood T cells that play a role in immune surveillance against cancer. Also, because $\gamma\delta$ T cells recognize molecules directly expressed by cancer cells without antigen presenting cells, such as dendritic cells (DCs), in a major histocompatibility complex (MHC)-unrestricted manner, they have the potential to be novel immunotherapeutic tools against cancer. Human $\gamma\delta$ T cells were recently used in cancer immune therapy due to increased cytotoxicity. MHC class I expression in prostate cancer is often down-regulated [9] and is an obstacle in DC-based immune therapy. Therefore, $\gamma\delta$ T cell immunotherapy is a suitable option in prostate cancer treatment. $\gamma\delta$ T cells recognize cancer cells via interactions between natural killer group 2 member D protein (NKG2D) on $\gamma\delta$ T cells and NKG2D ligands on cancer cells (e.g., MHC class I chain-related gene A/B [MIC-A/B]) [10]. MIC-A/B is minimally expressed in normal cells, but frequently expressed in cancer cells of epithelial origin, leading to high $\gamma\delta$ T cell affinity to cancer cells. After target cell recognition, $\gamma\delta$ T cells express cytotoxic molecules, such as perforin and granzymes, against the target. Perforin penetrates cell membranes and forms pores on the cell surface that allow granzyme proteases to enter, resulting in cytotoxicity [11].

Also, another issue for Ad based gene therapy is; viral infections due to Ad5 are endemic in most human populations, and the majority of humans have pre-existing humoral and/or cellular immunity to Ad5, which could severely limit use of Ad5-based vectors in gene therapy

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Figure 1: Characteristics of Ad-mediated gene delivery systems. (a) Reduced CAR expression in cancer cells is an obstacle in Ad vector infection. (b) Ad5-specific immunity of the host can serve as an obstacle in Ad vector infection. (c) Ad vector carrier systems using $\gamma\delta$ T cells may circumvent these obstacles.

[12]. Developing novel Ad vectors that can circumvent the suppressive effects of pre-existing anti-Ad5 immunity is an important research priority [13]. In contrast to Ad5, Ad35 is an alternative adenoviral serotype to which the majority of humans do not have neutralizing antibodies. Sumida et al. showed that rAd serotype 35 (rAd35) vector-based vaccines elicited potent cellular immune responses that were not significantly suppressed by anti-Ad5 immunity [13-15]. Additionally, the incidence of anti-Ad35 in humans has been reported to be less than 20% [12,16]. However, Ad5-specific antibodies against multiple capsid components have been reported [17], and Ad5-specific neutralizing antibodies (NAbs) have been shown to be directed primarily against the Ad5 hexon protein in humans [13], which is another obstacle in Ad5/F35 vector gene transduction (Figure 1).

Materials and Methods

Human $\gamma\delta$ T cell preparation

Human peripheral blood mononuclear cells (PBMCs) were isolated by Ficoll-Hypaque density gradient separation. After separation, PBMCs were cultured in RPMI-1640 medium (Wako, Osaka, Japan) supplemented with 10% autologous serum and stimulated with 1 μ M ZOL and 100 IU/mL IL-2 on day 0. Three quarters of the medium was replaced with AlyS505N medium (Cell Science & Technology Institute, Miyagi, Japan) supplemented with 2 mM L-glutamine (Wako) every three days [18]. After 14 days in culture, maturation of expanded $\gamma\delta$ T cells was evaluated by flow cytometry.

Adenovirus vector preparation

Transduction efficiency of Ad5/F35-GFP chimeric types 5 and 35 fiber proteins expressing green fluorescent protein (GFP) driven by the cytomegalovirus promoter was tested. Recombinant adenoviruses were purified by Adeno-X[™] Maxi Purification Kit (Clontech Laboratories, CA, USA), and stored in aliquots at -80°C . Viral stocks were titrated using Adeno-X[™] Rapid Titer Kit (Clontech Laboratories).

Optimization of infection conditions

$\gamma\delta$ T cells were infected with Ad5/F35-GFP at 100, 300, and 500 pfu/cell and incubated at 37°C in a humidified atmosphere with 5% CO_2 for

48 h. A subsample of $\gamma\delta$ T cells infected with Ad5/F35-GFP at 300 pfu/cell were incubated with 10% glovenin-I (Immunoglobulin G, Nihon Pharmaceutical Co., Tokyo, Japan) every 4 h to remove unadsorbed viruses. After 48 h, $\gamma\delta$ T cells were analyzed by flow cytometry (FACS Caliber, Becton Dickinson) with anti-TCR $\gamma\delta$ -FITC, anti-TCR $\gamma\delta$ -APC, anti-NKG2D-PE, anti-CD25-PECy7 (BD Biosciences, San Diego, CA), anti-CD3-PerCP (BioLegend, San Diego, CA), anti-CAR-FITC (Santa Cruz Biotechnology, Santa Cruz, CA), anti-CD46-FITC (AbD Serotec, Raleigh, NC), and for GFP expression.

Production of carrier cells

$\gamma\delta$ T cells were infected with Ad5/F35 at 300 pfu/cell and incubated at 37°C in a humidified atmosphere with 5% CO_2 for 16 h. After infection, unadsorbed viruses were removed by adding 10% glovenin-I to the culture medium for 1 h and washing twice with fresh medium.

Cell lines and co-culture assays

Prostate cancer cell line PC3 was obtained from American Type Culture Collection (Manassas, VA, USA). Cells were cultured in RPMI-1640 medium (Wako) containing 10% fetal bovine serum, streptomycin (100 $\mu\text{g}/\text{mL}$), and penicillin (100 U/mL). PC3 cell surface receptors were analyzed by flow cytometry with anti-MIC A-PE (R&D Systems, Minneapolis, MN), anti-MIC B-APC (R&D Systems), anti-CAR-FITC, and anti-CD46-FITC. PC3 cells (target) were incubated in triplicate with carrier cells (effector) at an effector: target (E:T) ratio of 20:1 for 48 h with and without 10% glovenin-I. After 48 h, PC3 cells were analyzed by flow cytometry with anti-CD45-PerCP (BioLegend), anti-EpCAM-APC (BioLegend), and for GFP expression.

Infection status

GFP expression in $\gamma\delta$ T cells and PC3 cells transduced with Ad5/F35-GFP was determined using a BIOREVO BZ-9000 microscope (Keyence, Osaka, Japan).

PC3 cells were incubated with Ad5/F35-infected $\gamma\delta$ T cells to determine cellular localization of internalized Ad5/F35 particles. PC3 cells were harvested after 48 h in co-culture and processed for electron microscopy.

Results

Expansion of $\gamma\delta$ T cells

Fluorescent activated cell sorting (FACS) analysis revealed 94.3% purity of $\gamma\delta$ T cells from PBMCs after stimulation with IL-2 and zoledronic acid (ZOL) in culture for 14 days.

CAR and CD46 expression

CD46 and CAR expression levels were examined in $\gamma\delta$ T cells. CAR expression in $\gamma\delta$ T cells was minimal. In contrast, more than 90% of $\gamma\delta$ T cells expressed CD46 (Figure 3). Based on these results, $\gamma\delta$ T cells are likely capable of being efficiently infected with Ad5/F35-GFP.

Ad5/F35-GFP infection and time in co-culture

Ad5/F35-GFP infection rates in $\gamma\delta$ T cells were investigated. After co-culturing $\gamma\delta$ T cells and Ad5/F35-GFP for 48 h, approximately half of the $\gamma\delta$ T cells were infected with Ad5/F35-GFP at concentrations ranging from 300 to 500 MOI (Figure 4a). $\gamma\delta$ T cell GFP expression was confirmed by flow cytometry (Figure 4a) and fluorescent microscopy (Figure 4b). The maximum infection rate was observed after 16 h in culture (Figure 5a), which was determined as the optimal co-culture time based on an Ad5/F35-GFP concentration of 300 MOI. Usually it takes about 15 to 20 minutes for adenovirus infects to the target cell. However, when adenovirus infects to $\gamma\delta$ T cells it might be an obstacle that, interferon-gamma is released from activated $\gamma\delta$ T cells and gives harmful influence to Ad vector. This might be a reason that

Figure 2: Ad delivery system strategy showing high affinity between Ad5/F35 vectors and $\gamma\delta$ T cells, also Ad5/F35-infected $\gamma\delta$ T cells and cancer cells, resulting in a high rate of gene delivery to cancer cells.

Figure 3: Minimal CAR expression in $\gamma\delta$ T cells. Over 90% of CD46 expression on $\gamma\delta$ T cells was confirmed. Based on these results, $\gamma\delta$ T cells can likely be infected with Ad5/F35-GFP.

Figure 4: After 48 h in co-culture, approximately half of the $\gamma\delta$ T cells were infected with Ad5/F35-GFP at concentrations ranging from 300 to 500 MOI. GFP expression in $\gamma\delta$ T cells as confirmed by flow cytometry (a) and fluorescent microscopy (b). The P values shown were calculated by post-hoc test.

Figure 5: Maximum Ad5/F35-GFP infection rate in $\gamma\delta$ T cells was observed after 16 h in culture (a). Activation markers (NKG2D and CD25) in $\gamma\delta$ T cells were preserved during co-culture with Ad5/F35-GFP. Over 40% of $\gamma\delta$ T cells were infected with Ad5/F35-GFP (b). The P values shown were calculated by Student's t-test. The whiskers represent data range.

appropriate co-culture time is considerably long (16 h) and appropriate concentration of Ad vector is considerably high (300 MOI).

Expression of NKG2D, an activating or co-activating receptor expressed on $\gamma\delta$ T cells, and CD25, an activation marker for $\gamma\delta$ T cells, was maintained in $\gamma\delta$ T cells (data not shown). Under these conditions, over 40% of $\gamma\delta$ T cells were infected with Ad5/F35-GFP (Figure 5b).

MIC A/B, CD46, and CAR expression in PC3 cells

MIC A/B is a key molecule in cancer cell recognition by the NKG2D ligand in $\gamma\delta$ T cells. CD46 is a key molecule for F35 in Ad5/F35 for infection. Expression of MIC A/B and CD46, but not CAR, in target PC3 cells was confirmed by flow cytometry (data not shown).

Ad5/F35-GFP infection in PC3 cells

After MIC A/B and CD46 expression in PC3 cells was confirmed, Ad5/F35-GFP-infected $\gamma\delta$ T cells were co-cultured with PC3 cells for

48 h to determine whether $\gamma\delta$ T cells transported Ad5/F35-GFP into the cells.

Glovenin-I was utilized as a substitute for anti-Ad antibody. Because we wanted to confirm whether $\gamma\delta$ T cells can deliver Ad vector to cancer cells under the condition of anti-Ad immunity, Glovenin-I was utilized for this experiment.

Without Glovenin-I (glovenin(-)), approximately 100% of PC3 cells expressed GFP. Over 50% of PC3 cells expressed GFP in the presence of Glovenin-I (glovenin(+)) (Figure 6a). GFP luminescence within PC3 cells was confirmed using fluorescent microscopy (Figure 6b).

GFP gene transduction rates using $\gamma\delta$ T cells were significantly higher than in PBMCs and natural killer cells (NKs), with a 7-fold increase compared to NKs (data not shown).

Ad vector particles were observed within endocytic vesicles (Figure

Figure 6: After co-culturing Ad5/F35-GFP-infected $\gamma\delta$ T cells and PC3 cells for 48 h, the rate of Ad5/F35-GFP transport into PC3 cells was evaluated. Without glovenin, approximately 100% of the PC3 cells expressed GFP. Over 50% of PC3 cells expressed GFP in the presence of immune-globulin (a). GFP luminescence within PC3 cells was confirmed using fluorescent microscopy (b). The P values shown were calculated by post-hoc test. The whiskers represent data range.

Figure 7: To determine cellular localization of internalized Ad5/F35 particles, PC3 cells were evaluated using electron microscopy. Ad vector particles were observed within endocytic vesicles (a) (white arrow indicates $\gamma\delta$ T cells, black arrow indicates PC3 cells). Ad vectors were observed between adjacent $\gamma\delta$ T cells and PC3 cells (b) (white arrows indicate Ad vectors). Multivesicular bodies (MVBs) were observed within the cytoplasm of PC3 cells (black arrow), and Ads were recognized within MVBs (white arrow) (c).

7a1-3). Ad vector particles were recognized between adjacent $\gamma\delta$ T and PC3 cells (Figure 7a1-3). Multivesicular bodies (MVBs) were observed within the cytoplasm of PC3 cells, and Ads were recognized within MVBs (Figure 7b).

Discussion

Adenovirus-mediated gene therapy for prostate cancer has recently been studied [19,20]. However, definitive therapeutic effects of this treatment are still unknown. We explored a new methodology of delivering genes to target cells using a fiber modified Ad vector (Ad5/F35) within $\gamma\delta$ T cells and demonstrated $\gamma\delta$ T cells as potential carriers of the vector for transporting genes into cancer cells. The results of this study may lead to increased Ad vector-based gene transduction efficacy.

Therapeutic forms of Ads are currently being developed to treat human diseases, including cancer. Ads are now considered promising

potential vectors for gene delivery. However, conventional methods that deliver naked genes across cell membranes are not clinically efficient. Since the first Ad vector-based gene therapy method was introduced in 1989, many vector types have been developed that overcome lower gene induction rates into the target cell. In addition to efficient Ad vectors, it is also critical to establish efficient Ad vector carrier technology.

A critical issue has been to increase Ad vector infection rates in target cells. Viral infection is mediated by a specific receptor on target cells. Recent studies have demonstrated drawbacks to using conventional Ad5 vectors, including poor rates of infection in cells that lack the primary CAR. Unfortunately, CAR expression is often down-regulated in many types of cancer [21], including prostate cancer. The Ad5/F35 vector has improved infection rates in target cells with down-regulated CAR expression. However, CD46 rather than CAR expression in target cells is necessary for infection with Ads containing

type 35 fiber protein. Strong CD46 expression was recognized in $\gamma\delta$ T cells. Therefore, the Ad5/F35 vector is suitable for targeting and infecting $\gamma\delta$ T cells. After infection, $\gamma\delta$ T cells can carry the Ad5/F35 vector into cancer cells by evading anti-Ad5 immunity in the host for safe transfection of the loaded gene. $\gamma\delta$ T cell affinity for cancer cells is generally high due to interactions between NKG2D in $\gamma\delta$ T cells and MIC A/B in cancer cells. Therefore, we hypothesized that $\gamma\delta$ T cells could be “vehicles” for Ad5/F35 delivery (Figure 2).

High gene transduction rates using $\gamma\delta$ T cells may be related to high $\gamma\delta$ T cell affinity for cancer cells, which could be due to preservation of NKG2D expression in Ad5/F35-GFP-infected $\gamma\delta$ T cells during co-culture (Figure 5b). Ad5/F35-GFP did not negatively influence NKG2D expression in $\gamma\delta$ T cells. Although there was an infection rate error, approximately half (40% to 60%) of all $\gamma\delta$ T cells were infected with Ad5/F35-GFP (data not shown). This infection rate is likely sufficient because the GFP gene was efficiently delivered to PC3 cells.

Anti-Ad5 immunity in patients is a serious drawback. The majority of adults have neutralizing antibodies to Ad5 [22]. Ad5-specific NABs primarily directed against the Ad5 hexon protein are also obstacles for gene transduction to cancer cells using the Ad5/F35 vector. Because the Ad5/F35 vector hexon protein originated in Ad5, Ad5-specific NABs bind to the Ad5/F35 hexon. Even if the Ad5/F35 vector is used for conventional gene therapy, circumventing interrupted gene transduction caused by Ad5-specific NABs is difficult. Allowing Ad5/F35 vectors to reach to cancer cells by circumventing Ad5-specific NABs is a reasonable approach. If Ad5/F35 vectors can be carried to cancer cells using a “delivery vehicle”, it may be possible to decrease Ad5-specific NAB interference in gene transduction. To confirm this hypothesis, Ad5/F35-GFP-infected $\gamma\delta$ T cells (Ad5/F35-GFP loading delivery vehicle) were co-cultured with PC3 cells. The GFP gene was efficiently transduced into PC3 cells using the $\gamma\delta$ T cell “vehicle”. Even in the presence of glovenin-I (glovenin-I was used as a substitute for anti-Ad antibody), GFP expression was observed in approximately 60% of target PC3 cells. In the absence of glovenin-I, GFP expression was observed in almost all target PC3 cells. These results could indicate that $\gamma\delta$ T cells function as a shield for Ad5/F35-GFP against anti-Ad immunity.

We hypothesized that “pore forming proteins” assist in gene transduction from $\gamma\delta$ T cells to PC3 cells. Perforin creates pores on the cell surface that may facilitate internalization of naked genes into cancer cells. We measured perforin and CD107a (also known as lysosomal-associated membrane protein-1) expression levels in $\gamma\delta$ T cells before and after co-culture with Ad5/F35-GFP. Infection with Ads induced expression levels of both pore forming proteins in $\gamma\delta$ T cells (data not shown).

If only GFP genes were transported into PC3 cells, there should be no differences in GFP gene induction rates into PC3 cells between the different culture settings (glovenin(+) and glovenin(-)). However, gene induction rates differed between the two conditions. The GFP transduction rate of glovenin(-) was significantly higher than glovenin(+) (approximately 100% vs. 60%, respectively) (Figure 6a). Differences in infection rates were assumed to be related to a “ripple effect” in that the Ad5/F35-GFP vector was released by PC3 cells that were disrupted by $\gamma\delta$ T cells. Secondary Ad5/F35-GFP vector infection of neighboring PC3 cells could then be induced. To confirm this hypothesis, we investigated the presence of Ad5/F35-GFP vectors within PC3 cells using electron microscopy. We demonstrated GFP expression and the presence of Ad5/F35-GFP vectors within cancer

cells as confirmed by fluorescent microscopy and electron microscopy, respectively (Figure 7).

The present study demonstrates that $\gamma\delta$ T cells are novel, effective transporters of Ad5/F35 vectors into cancer cells. This carrier system may improve low gene transduction rates and could result in increased efficacy of Ad5/F35 vector-based gene therapy. To clarify the anti-cancer efficacy of this novel carrier system, additional research using Ad5/F35 vectors containing therapeutic genes is necessary.

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