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Establishment of a mouse melanoma model system for the efficient infection and replication of human adenovirus type 5-based oncolytic virus

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ABSTRACT

Due to poor adenoviral infectivity and replication in mouse tumor cell types compared with human tumor cell types, use of human-type adenoviral vectors in mouse animal model systems was limited. Here, we demonstrate enhanced infectivity and productive replication of adenovirus in mouse melanoma cells following introduction of both the Coxsackievirus and adenovirus receptor (CAR) and E1B-55K genes. Introduction of CAR into B16BL6 or B16F10 cells increased the infectivity of GFP-expressing adenovirus; however, viral replication was unaffected. We demonstrated a dramatic increase of adenoviral replication (up to 100-fold) in mouse cells via E1B-55K expression and subsequent viral spreading in mouse tissue. These results reveal for the first time that human adenovirus type 5 (Ad5)-based oncolytic virus can be applied to immunocompetent mouse with the introduction of CAR and E1B-55K to syngenic mouse cell line.

1. Introduction

Human Ad5-based adenoviral vectors have been used frequently in cancer gene therapy [1,2]. Oncolytic adenoviruses used in therapeutics are engineered to replicate and induce cancer cell lysis selectively [3]. To achieve cancer cell selectivity, two major strategies have been employed. One is the deletion of viral genes (the E1B-55K gene or a 24-bp deletion of the E1A gene) essential for replication [4,5]. The other strategy is to insert tumor- or tissue-specific promoters to control viral replication [6,7].

Before clinical trials of such adenoviral vectors, it is imperative to confirm the efficacy and toxicity of adenoviral vectors expressing therapeutic genes in an animal model system. Because adenoviral infectivity and replication is limited to species-specific cells [8–10], and thus, minimal to no adenovirus replication in mouse exception of mouse epidermal cells with unknown mechanism [11–14], most animal studies of adenoviral gene therapies have been examined in immunodeficient mice bearing human xenograft tumors [9]; however, one of the most substantial limitations to this approach is that the immunodeficient mice are not representative of the host immune response, preexisting immunity to adenovirus administration, or the efficacy of immune gene(s)-armed adenoviral vectors. Recently, a Syrian hamster animal tumor model was developed to study oncolytic Ad5-based vectors, since the Syrian hamster is permissive for human adenovirus replication in normal tissues as well as in Syrian hamster cancer cells [8,10]. In general though, a lack of hamster-specific reagents persists despite the development of more established hamster cancer cell lines [10]. As a result, no suitable immunocompetent mouse model for testing human adenoviral vector is currently available. Thus, we developed a modified mouse cancer cell line that can be easily infected by human adenoviral vectors and supports replication by these vectors.

Ad5, which is the primary vector administered to most patients, uses the Coxsackievirus and adenovirus receptor (CAR) for binding on the target cell surface [15–17], and Ad5 infection is initiated by the high-affinity binding of the fiber knob to the CAR [18,19], followed by internalization mediated by the binding of arginine-glycine-aspartic acid (RGD) motifs in the penton base to

Abbreviations: GFP, green fluorescence protein; CMV, cytomegalovirus; PBS, phosphate buffered saline; PFU, plaque-forming unit; HRP, horseradish peroxidase.

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integrins $\alpha_v\beta_3$ and $\alpha_v\beta_5$ on the cell surface [20]. Ad5 or Ad2 binds efficiently to murine CAR as well as to human CAR, because the sequences are highly conserved between species [21]. CAR expression in adult mice, however, is restricted to fewer cell types [21,22].

Most cases reported, to date, have described poor adenoviral replication in mouse cells due to decreased levels of early gene products, such as E1A or E1B [14]. E1A is the first viral gene that is expressed when the viral genome reaches the nucleus [23]. The main functions of the E1A gene product are to activate transcription of other early genes and to bind to Rb, resulting in release of E2F, which is critical for the activation of the adenovirus E2 gene expression cassette and transcriptional activation of cell cycle S-phase entry genes to generate a favorable cellular environment for viral replication [24–26]. The E1B gene products (19K and 55K proteins) are involved in prolonging cell survival by inhibiting E1A-induced apoptosis (E1B-19K) [27,28], in viral replication, in transport of viral mRNAs to the cytosol from the nucleus, and in targeting p53 for destruction (E1B-55K) [26,29–31].

In the present study, we demonstrated that mouse melanoma cells became permissive to human adenoviral replication following introduction of both CAR and E1B-55K.

2. Materials and methods

2.1. Cell culture

The mouse melanoma cell lines B16BL6, B16F10 and human melanoma cell line A375 were cultured in Dulbecco's modified Eagle's medium with 10% fetal bovine serum (HyClone, Logan, UT, USA). DU-145, a human prostate adenocarcinoma and 293A, a subclone of the human embryonic kidney 293 cell (Invitrogen, Carlsbad, CA, USA) were also cultured in Dulbecco's modified Eagle's medium with 10% fetal bovine serum (HyClone, Logan, UT, USA). All cells were maintained at 37 °C in a humidified atmosphere containing 5% CO_2.

2.2. Reagents

Antibodies to E1A and actin were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA), and the antibody to CAR was purchased from Thermo Fisher Scientific (Waltham, MA, USA). The anti-Ad5 antibody was purchased from Abcam (Cambridge, UK). Trizol was purchased from Life Technologies (Carlsbad, CA, USA), and all other chemicals were purchased from Sigma–Aldrich (St Louis, MO, USA).

2.3. Cloning of CAR

The CAR-expressing pCDNA3.1hygro was constructed for stable transfection into mouse melanoma cells in order to generate cell lines with higher infectivity. For the cloning of CAR, RNA was extracted from 293A, and the CAR cDNA was generated via reverse transcription using reverse transcriptase and subsequent PCR, which was performed using the following primers: sense primer, 5′-ATGGCGCTCCTGCTGTGCTTC-3′ and antisense primer, 5′-CTATACTATAGACCCATCCTTG-3′. PCR conditions were as follows: 95 °C; 34 cycles of 95 °C 30 s, 55 °C 30 s, and 72 °C 1 min 30 s; and 72 °C 5 min. Next, the PCR product was inserted into the pDrive cloning vector (Qiagen, Valencia, CA, USA). Next, following *Hin*d III/*Bam*H I digestion, the PCR product was subcloned into *Hin*d III/*Bam*H I-digested pcDNA3.1hygro to yield pcDNA3.1hygro-CAR.

2.4. Subcloning of E1A or CAR and E1B-55K

The adenoviral E1A gene was originated from pBSK[3484], as described in detail by Kim et al. [32] This plasmid consists of inverted terminal repeats (ITR), a packaging signal, the mouse survivin promoter, E1A-BGH polyA-E1B, the 55K gene cassette, and the Ad E1 right region. It was used as a template for the PCR amplification of the E1A gene using the sense primer 5′-CGC GGATCCATGAGACATATTATCTGCCACG-3′ and the antisense primer 5′-TGCTCTAGATCCCCAGCATGCCTGCTATTG-3′. After digestion with *Bam*H I/*Xba* I, the EIA PCR product was inserted into *Bam*H I/*Xba* I-digested pcDNA3.1-hygro to yield pcDNA3.1hygro-E1A. For the construction of both CAR and E1B-55K, the pIRES vector (Clontech, Mountain View, CA, USA) was used. The CAR cDNA described above was used as a template for the PCR, which was performed with the same conditions described above with the following primers: sense primer 5′-ATAGCTAGCATGGCGCTCCTGCTGTGCTT-3′ and antisense primer 5′-GCGCGAATTCCTATACTATAGACCCATCC-3′. Then, the PCR product was inserted into the pIRES cloning vector. Next, following *Nhe* I/*Eco*R I digestion, the CAR gene fragment was subcloned into *Nhe* I/*Eco*R I-digested pIRES to yield pIRES-CAR. Next, for E1B-55K subcloning, pBSKII-3484, which contains both E1A and E1B-55K DNA sequences, was used as a PCR template. The PCR reaction was performed under the PCR conditions described above using the following primers: sense primer 5′-ACTGTCGACATGGAGCGAAGAAACCCATC-3′ and antisense primer 5′-ATAGCGGCCGCTCAATCTGTATCTTCATCG-3′. Then, the PCR product was inserted into the pIRES-CAR construct. Next, following *Sal* I/*Not* I digestion, the E1B-55K gene fragment was subcloned into *Sal* I/*Not* I-digested pIRES-CAR to yield pIRES-CAR-E1B55K.

2.5. Construction of GFP-expressing recombinant adenovirus

The adenovirus vector vmdl324Bst containing the Ad5 genome with a deletion in the E1 region (340–4640 nucleotides of Ad5) and the E3 region (28,592–30,470 nucleotides of Ad5) as well as the IX gene was linearized with *Bst*B I for homologous DNA recombination in *Escherichia coli* BJ5183 cells. To generate GFP-expressing adenovirus, subcloning of the GFP gene from pEGFP-N1 into pCA14 adenovirus shuttle vector (Microbix, Mississauga, Ontario, Canada) was performed after digestion with *Xho* I-*Xba* I. Next, homologous recombination was performed in BJ5183 cells following digestion of the shuttle vector with *Xmn* I and *Bst*B I-linearization of the vmdl324Bst adenovirus vector. The appropriate homologous recombinant adenoviral plasmid DNA was digested with *Pac* I and transfected into 293A cells to generate GFP-expressing adenovirus. These adenoviruses with or without the GFP gene were propagated in 293A cells and amplified for purification according to standard methods. Titration was performed by estimating the infectious viral particles with a standard plaque assay kit developed by Qbiogene (Carlsbad, CA, USA) in 293A cells.

2.6. Construction of replication-competent adenovirus (RCA) or oncolytic adenoviral vectors

For the construction of adenoviral vectors, a synthetic gene (3484 base pair) was used as a backbone construct for the shuttle vector, and the DNA was pBSK[3484] as described above. The mouse survivin promoter controlling E1A was replaced with the CMV promoter, and pcDNA3.1hygro was used as a template for PCR amplification of the CMV promoter using the sense primer 5′-CGGGGTACCGATGTACGGGCCAGAT-3′ and the antisense primer 5′-CCGCTCGAGAATTTCGATAAGCCAG-3′. After digestion of the PCR product with *Kpn* I/*Xho* I, the CMV promoter was inserted into *Kpn* I/*Xho* I-digested pBSK[3484]. The pBSK[3484] construct was again digested with *Fsp* I and *Bam*H I and ligated into *Ssp* I/*Bgl* II-digested pCA14 to generate the final E1 shuttle vector. The resultant pCA14-[3484] shuttle vector was linearized by *Xmn* I digestion, and the linearized pCA14-[3484] was cotransformed into *E. coli* BJ5183 together with *Bsp*1191-digested vmdl324Bst for

homologous recombination. The homologous recombinant adenoviral plasmid DNA was digested with *Pac* I and transfected into 293A cells to generate an artificial RCA Ad-3484-CMV-E1A-ΔE1B19. To produce oncolytic adenovirus, the E1B-55K gene cassette was deleted by *Eco*R I and *Sal* I digestion followed by blunting. This type of pBSK[3484]-ΔE1B55 construct was again digested with *Fsp* I and *Bam*H I and ligated into *Ssp* I and *Bgl* II-digested pCA14 for the formation of the final E1 shuttle vector. The resultant pCA14-[3484]-ΔE1B55 shuttle vector was linearized by *Xmn* I digestion, and the linearized pCA14-[3484]-ΔE1B55 was cotransformed into *E. coli* BJ5183 together with *Bst*B I-digested vmdl324Bst for homologous recombination. The homologous recombinant adenoviral plasmid DNA was digested with *Pac* I and transfected into 293A cells to generate the tumor-selective, replication-competent Ad-3484-CMV-E1A-ΔE1B19/55.

2.7. Oncolytic assay for cytopathic effect (CPE)

To compare the CPE of tumor-selective, replication-competent adenoviruses (Ad-3484-E1A-ΔE1B19/55), 293A cells, B16BL6-CAR, or B16BL6-CAR-E1B55 were first grown to ~80% confluency in 24-well plates, and then infected at various MOIs (multiplicities of infection) of adenoviruses. After 8 h of infection, cells were monitored daily under a microscope. When the infected 293A cells exhibited cell lysis at the lowest MOI, the remaining cells on the plate were fixed with 4% paraformaldehyde and stained with 0.5% crystal violet.

2.8. E1B-55 K polyclonal antibody production

A 25-mer synthetic peptide, MERRNPSERGVPAGFSGHASVESGC, from N-terminal of E1B-55K was selected among 5 candidates and conjugated to BSA as an immunogenic carrier protein and used to immunize two New Zealand White rabbits. The overall processes including antibody production and purification were performed by Young In Frontier (Seoul, Korea).

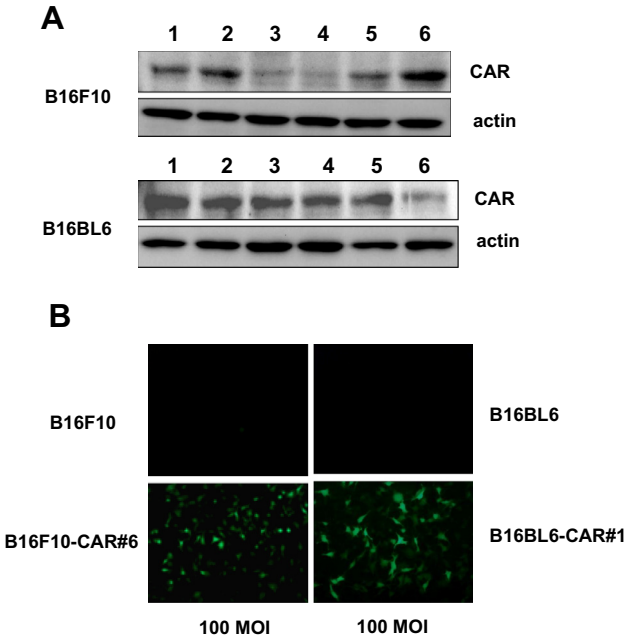

Fig. 1. Infectivity of adenovirus expressing GFP increased in CAR-expressing mouse melanoma cells. Mouse melanoma cells (B16BL6 and B16F10) were stably transfected with pcDNA3.1hygro-CAR and selected for expression of a clone expressing CAR maximally in each cell line (A). B16BL6/B16F10 or B16BL6-CAR/B16F10-CAR cells were infected with replication-incompetent adenovirus expressing GFP at an MOI of 100 (B).

2.9. Immunohistochemistry (IHC)

Seven days after subcutaneous injection of B16BL6 or B16BL6-CAR-E1B55K tumor cells (5×10^5) into the abdominal region of C57BL/6 male mice, the mice were infected with oncolytic adenovirus (Ad-3484-CMV-E1A-ΔE1B19/55, 1×10^9 PFU/100 μl) intratumorally three times every other day. Seven days after the last viral injection into each mouse, tumor tissues were extracted, fixed and embedded in paraffin for IHC staining. IHC was performed as described here. Tissue section slides were deparaffinized twice with xylene for 10 min each, and then rehydrated using a graded alcohol series. After removing endogenous peroxidase using 0.1% H_2O_2, slides were washed three times with PBS. Antigen retrieval

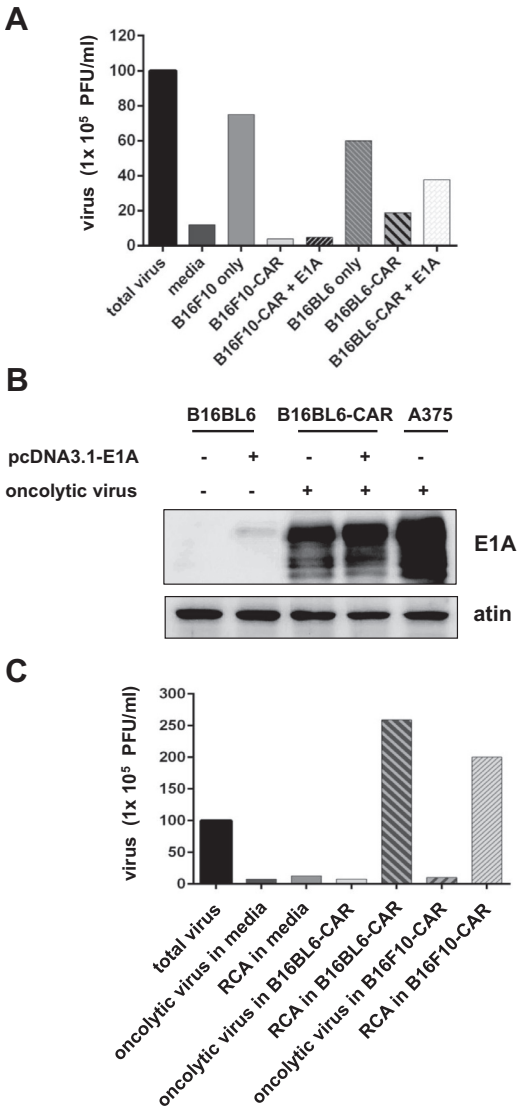

Fig. 2. E1A expression does not affect adenoviral replication in mouse melanoma cells expressing CAR. Virus titration was examined after infection of pcDNA3.1-E1A-transfected B16F10 (or B16BL6) or B16F10-CAR (or B16BL6-CAR) cells with oncolytic adenovirus (Ad-3484-CMV-E1A-ΔE1B19/55) at 1×10^7 PFU/ml (initial total virus) for their capability to produce progeny virus after two days (A). B16BL6 or B16BL6-CAR cells were transfected first with pcDNA3.1-E1A and then infected with oncolytic adenovirus at an MOI of 50. A375, which served as a positive control, was also infected with the same virus at an MOI of 50. After two days of infection, protein lysates were subjected to immunoblotting for the detection of E1A (B). Virus titration was also examined after infection of B16F10-CAR or B16BL6-CAR cells with oncolytic adenovirus or replication-competent adenovirus (Ad-3484-CMV-E1A-ΔE1B19) at 1×10^7 PFU/ml (initial total virus) for their capability to produce progeny virus after two days (C).

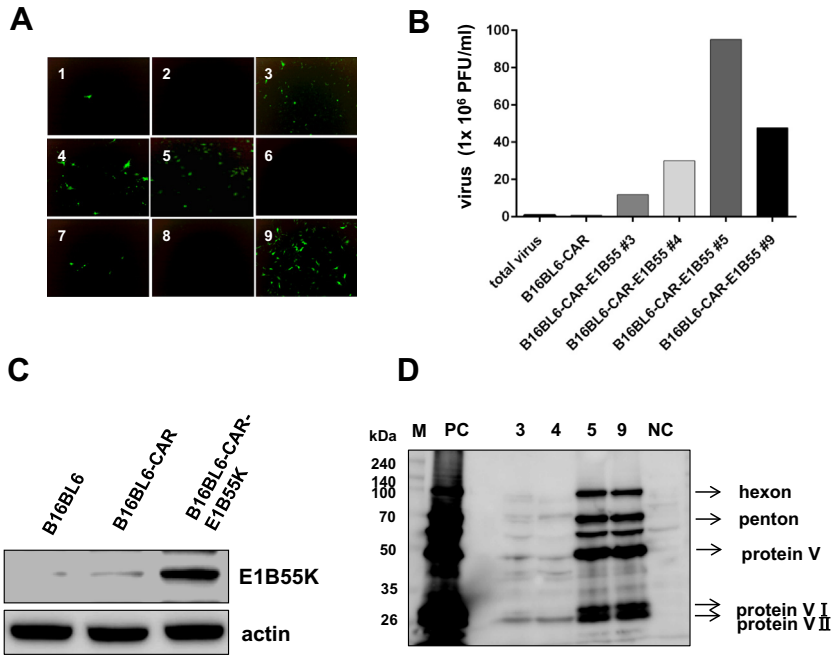

Fig. 3. Enhanced adenoviral replication was observed in mouse melanoma cells expressing both CAR and E1B-55K. After stable transfection of B16BL6 cells with pIRES-CAR-E1B55K, nine clones were selected. Next, GFP expression was examined after infection with defective adenovirus expressing GFP (A). Four positive selected clones (#3, #4, #5, and #9), which had higher GFP expression, were also examined for their capability to produce progeny virus after two days of infection of cells with oncolytic adenovirus at 1×10^6 PFU/ml (initial total virus) (B). E1B-55K protein was detected by using E1B-55K polyclonal antiserum from selected clone #5 of B16BL6-CAR-E1B55K cell line (C). Levels of adenoviral late gene products were examined via immunoblotting after infection with oncolytic adenovirus (Ad-3484-CMV-E1A-ΔE1B19/55) at an MOI of 50 for two days. For the detection of late gene products, the Ad5 antibody was used. As a positive control (PC), DU-145, a human prostate cancer cell line, was used. The numbers 3, 4, 5, and 9 indicate the selected B16BL6-CAR-E1B55K clone, whereas NC denotes that B16BL6-CAR was used as a negative control (NC). M denotes protein size marker (D).

was performed using 10 mM citrate buffer (pH 6.0; DAKO, Glostrup, Denmark) and a microwave oven. Tissues were permeabilized with 0.5% PBX (0.5% Triton X-100 in PBS) for 30 min. For the detection of adenovirus, antibodies against Ad5 were used. To develop the colored product, a mixture of DAB (3,3′-diaminobenzidine) Plus Chromogen and DAB Plus Substrate (Thermo Fisher Scientific) was added for 5 min. After washing with PBS, 20% hematoxylin counterstain was added for 2–5 min to stain the nuclei. Finally, tissue slides were dehydrated in a graded alcohol series. After clearing twice in xylene, slides were coverslipped with mounting media (xylene:mount = 1:1) for microscopy.

3. Results

3.1. GFP-expressing adenovirus exhibited increased infectivity in mouse melanoma cells

To overcome the typically low infectivity of mouse cells by human Ad5, mouse melanoma cells (B16BL6 and B16F10 cells) were stably transfected with the CAR gene. After confirming the expression of this gene in mouse melanoma cells (Fig. 1A), we demonstrated significantly increased adenoviral infectivity from the selected clones (Fig. 1B). Despite these increases, however, viral replication was not increased in either of the CAR- expressing mouse melanoma cells (Fig. 2A). Thus, the possibility that the viral expression in B16BL6-CAR after oncolytic adenovirus infection was due to low E1A expression was examined following E1A gene transfection into B16BL6-CAR. Viral replication was not increased, however, despite dramatically enhanced E1A expression in B16BL6-CAR cells compared with B16BL6 cells (Fig. 2B). Therefore, the gene product responsible for viral replication in mouse cells remained unclear. To address this problem, an artificial replication-competent adenovirus with a CMV promoter-driven E1A and E1B without E1B-19K was used. Indeed, viral replication was increased to produce progeny virus more than twice compared to the initial amount of infected virus in both melanoma cells (Fig. 2C).

3.2. Introduction of E1B-55K into CAR-expressing mouse melanoma cells significantly increased viral replication

Based on the result of Fig. 2B, E1B-55K appears to be a strong candidate to be responsible for viral replication. Moreover, because E1B-55K is known to mediate the shutdown of host protein synthesis, which is thought to facilitate late viral protein production [33], the E1B-55K gene was introduced into B16BL6-CAR cells. We identified four selected clones (#3, #4, #5, and #9) that had higher infectivity compared to other selected clones (Fig. 3A), and two of these clones (#5, #9) produced 50–100-fold more progeny adenovirus than the initial amount of infected virus in B16BL6-CAR cells (Fig. 3B). The expression of E1B-55K protein in the structure of B16BL6-CAR-E1B55K was confirmed by using newly produced E1B-55K polyclonal antibody (Fig. 3C). As a result, the replication nearly coincided with the differential expression of late gene production (Fig. 3D). In the case of human cancer cells (DU145), significant amounts of late gene products were detected despite the E1B-55K deficiency, indicating that human cancer cells possess another strategy to promote export and translation of late viral RNAs [31,33]. For example, the cellular levels of certain proteins downregulated by E1B-55K, such as Daxx, which functions as an essential repressor of adenoviral replication, can remain very low in human cells compared to mouse cells despite E1B-55K deficiency [34,35]. This possibility is now under investigation.

3.3. Increased oncolytic adenoviral replication was observed in vitro and in vivo

We also observed efficient oncolysis induced by viral replication in B16BL6-CAR-E1B55K, which is a newly-generated B16BL6-CAR

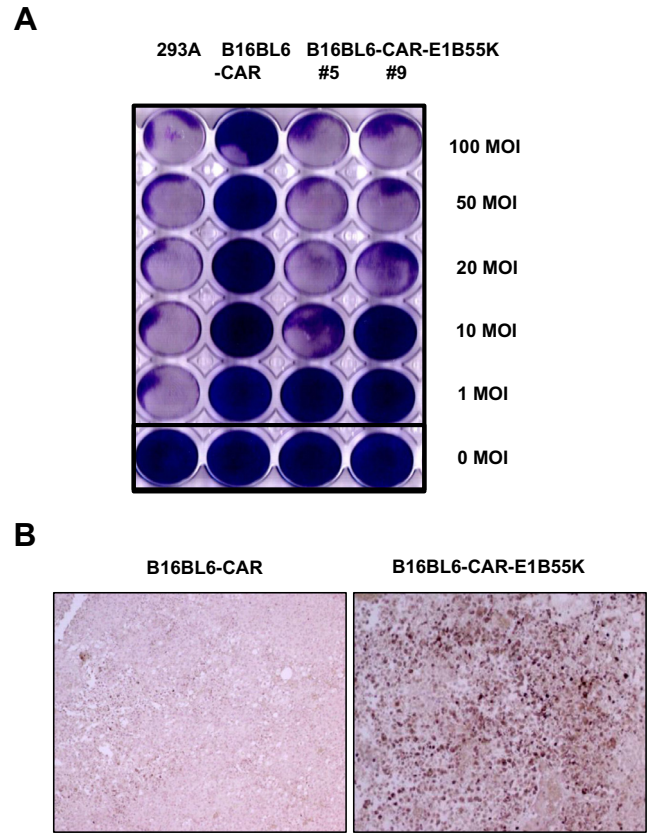

Fig. 4. Differential oncolytic activity and viral replication in B16BL6-CAR-E1B55K. To compare the oncolytic activity induced by Ad-3484-CMV-E1A-ΔE1B19/55, B16BL6 cells expressing CAR only or both CAR and E1B-55K or 293A cells were infected at various MOIs of 1–100. Immediately after the infected 293A cells at an MOI of 1 exhibited complete cell lysis, all remaining cells on the plate were fixed with 4% paraformaldehyde and stained with 0.5% crystal violet (A). Immunohistochemical analyses of oncolytic adenovirus in B16BL6 cells that express CAR and E1B-55K. After seven days of subcutaneous injection of B16BL6-CAR or B16BL6-CAR-E1B55K tumor cells (5×10^5) into the abdominal region of C57BL/6 male mice, the tumors were infected with oncolytic adenovirus (Ad-3484-CMV-E1A-ΔE1B19/55, 1×10^9 PFU/100 μl) intratumorally three times every other day. Seven days after the last viral injection, tumor tissues were extracted, fixed for 24 h in 10% formaldehyde, and embedded in paraffin for immunohistochemical (IHC) staining of adenovirus (B).

cell line expressing E1B-55K (Fig. 4A). Finally, adenoviral replication including viral spreading was clearly observed in tumor tissues originating from B16BL6-CAR-E1B55K implanted in syngenic mice (Fig. 4B). These results strongly suggest that adenoviral replication and subsequent viral spreading in immunocompetent mouse model system can be successfully applied by using a syngenic cell line expressing both CAR and E1B-55K.

4. Discussion

One of current requirements for the study of adenoviral gene therapy is to establish an optimal mouse model system other than using immunodeficient mice bearing human xenograft tumors [9,13]. In this study, we have shown that both of CAR and E1B-55K are indispensible for human type 5 adenoviral replication in mouse melanoma. E1A protein which is known to activate transcription of other early genes and to make cells more susceptible to viral DNA replication [26,36] was already highly expressed in B16BL6-CAR cells after oncolytic adenovirus infection into CAR-expressed mouse melanoma (Fig. 2B), suggesting that the cellular level of E1A after oncolytic adenovirus infection into CAR-expressing mouse melanoma could not limit the viral replication. This result was consistent with a previous study that reported no obvious correlation between E1A expression and ability to support virus replication [13]. However, based on the results of Figs. 2B and 3D that E1A protein and late gene products were highly expressed in human cancer cells compared to CAR and E1B-55K-expressing mouse cancer cells after infection of oncolytic adenovirus (Ad-3484-CMV-E1A-ΔE1B19/55), human-specific unknown factor(s) must be still necessary for optimal viral replication. Currently, the effort to find the unknown factor(s) is under investigation.

Taken together, our newly designed melanoma cell lines may be useful in mouse models especially for studying the effect of the host immune system on oncolytic virus-infected tumors [10]. This type of advance may ultimately expand the choices of animal model systems that are relevant to the given experimental condition.

Acknowledgments

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