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Characteristics of Oncolytic Adenovirus Replication and Gene Expression in Hypoxic Condition

Hong Sung Kim

Department of Biomedical Laboratory Science, Korea Nazarene University, Cheonan, Chung Nam 331-718, Korea

Adenovirus type 5 (Ad5) vectors have been used for gene transfer to a wide variety of cell types *in vivo* and *in vitro*. The advantages of adenovirus vectors include the high titer of virus readily obtained in large scale preparations, their ability to transduce dividing and non dividing cells, and the high level of transgene expression. Since adenovirus vectors do not integrate in host cell DNA, there is a lack of insertional mutagenesis. However, many human tumor cells lack expression of the adenovirus 5 receptors and contain areas of hypoxia. In order to identify the pattern of replication and gene expression of oncolytic adenovirus in hypoxic condition, multiple different fiber modified Ads (Ad5F/S11, Ad5F/S35, Ad5F/K7, Ad5F/K21, and Ad5F/RGD) was compared. The replication of all fiber modified adenovirus was inhibited in hypoxic condition in HEK 293 cells, but gene expression has variety on different tumor cell lines and the level of coxackievirus and adenovirus receptor (CAR) expression. These data suggest that CAR expression pattern and hypoxic condition of tumor are considered for optimal oncolytic adenovirus application.

Key Words: Adenovirus replication, Hypoxia, Gene expression

INTRODUCTION

It is well known that adenovirus type 5 is widely used in various gene therapies, but its gene expression in many human tumor cells is very low. The main reason of low gene expression is due to limited levels of the Ad5 receptor (predominately Coxsackie Adenovirus Receptor-CAR and integrins - αVβ 3 and 5) in many human tumor and receptor expression correlates with the efficiency of adenovirus mediated gene transfer (Jee et al., 2002). It is reported that a high multiplicity of infection may overcome limited CAR expression, but such high doses of Ad5 may be toxic (MacKenzie et al., 2000). Other strategies such as PEGylation, encapsulation in cationic liposome, and calcium phosphate precipitation, have helped increase the transduction efficiency of Ad5 to resistant cells but require additional manipulation of the cells. Recently, Modified adenoviral vector itself may be a promising method, since adenovirus target specificity is determined by the viral fiber and knob. Numerous of these strategies have been applied including the use of bi-specific antibodies and the Ad fiber knob (Blackwell et al., 1999; van der Poel et al., 2002), or of the genetic modification of the Ad fiber knob by incorporating targeting peptides such as polylysine (Garcia-Castro et al., 2001; Gonzalez et al., 1999). Also, it is possible to use fibers from naturally occurring serotypes of adenovirus (Ad11, Ad35) because of their different cellular tropisms.(Segerman et al., 2000; Stecher et al., 2001). In addition, it is well known that hypoxic areas of tumors are often resistant to chemo-radiotherapy, and cellular immunotherapy. It is reported that hypoxic region of tumor mass reduces adenovirus replication (Pipiya et al., 2005). In this study, we have checked the effect of hypoxia on virus replication and gene expression at various tumor cell lines using Ad5F/wt and the modified Adenovirus (Ad5F/S11, Ad5F/S35, Ad5F/K7, Ad5F/K21, and Ad5F/RGD).

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†Corresponding author: Hong Sung Kim, Department of Biomedical Laboratory Science 456 SSangyong, Seobuk, Chung Nam 331-718 Korea.
Tel: 041-570-4165, Fax: 041-570-4258
e-mail: hskim@kornu.ac.kr

MATERIALS AND METHODS

Cells

HEK293 (embryonic kidney), A549 and H1299 (human lung cancer), MDA-MB-435 (human breast tumor), and PC3 (human prostate tumor) were maintained in recommended medium; Dulbecco modified Eagle's medium (BioWhittaker, Walkersville, MD), RPMI-1640 (BioWhittaker, Walkersville, MD) or modified Eagle's medium (BioWhittaker, Walkersville, MD). Medias were supplemented with 10% fetal calf serum (Hyclone, Logan, UT), 100 unit/ml penicillin, and 100 μg/ml streptomycin (Life Technologies Inc., Gaithersburg, MD) and cells were maintained at 37℃, in 5% CO_2 in a humidified incubator.

Viruses

Ad5, Ad5F/S35, Ad5F/S11, Ad5F/K7, Ad5F/K21, Ad5F/RGD recombinant vectors were prepared and purified, and the titers were determined, as described previously (Brough et al., 1996; Wickham et al., 1996). Briefly, the fiber expression cassettes were introduced by recombination in an E1/E3-deleted Ad5 expressing Lac-Z. Each vector was produced by calcium phosphate transfection of HEK293 cells, followed by expansion of a single plaque. At the maximal cytopathic effect, the cells were harvested and pelleted. Vectors were extracted from the HEK293 cells by three consecutive freeze/thaw cycles and were amplified by infection of a larger culture of HEK293 cells. The vectors were purified by two cesium chloride gradient ultracentrifugation steps and desalted on exclusion columns (Bio-Rad Laboratories, Hercules, CA). The adenovirus vectors were stored at -70℃ in 10% glycerol, 10 mM Tris-Cl (pH 7.4), and 1 mM $MgCl_2$. The titer of the large-scale purified adenovirus preparation was determined by spectrophotometry and plaque assays on HEK293 cells. Preparations were routinely tested for replication-competent adenovirus (RCA) by plaque assays on A549 cells. All vectors used are named by their modification (Table 1).

Table 1. Fiber-modified adenovirus used in this study and their receptors

Fiber-modified Ad	Receptor
Ad5 (F/wt)	CAR/integrins
Ad5F/S11	CD46, CAR independent
Ad5F/S35	CD46, CAR independent
Ad5F/K7	Heparan sulfate
Ad5F/K21	Heparan sulfate
Ad5F/RGD	αV integrin

Ad: adenovirus, CAR: coxsackievirus and adenovirus receptor

Hypoxia

For this study, hypoxia is defined as 1% oxygen, which is approximately 7 mmHg. For all experiments, cells were grown on 6 well tissue culture dishes for 48 or 72 h at either ambient oxygen (20%) or 1% oxygen concentration with 5% CO_2. Hypoxia was established using a HERAcell 150 incubator, which uses N_2 and CO_2 gases to obtain a required oxygen concentration (Kendro Laboratory products, Newtown, CT, USA).

Flow cytometry

Tumor cells were incubated under normoxia or hypoxia. After 48 h, the cells were harvested and washed two times in PBS. Cells were incubated with primary antibody on 4℃ for 30 min, and washed with PBS twice. Cells were then incubated with FITC-goat-anti-mouse IgG for 30 min, and then washed with PBS twice. Cells were resuspended with 200 μl of PBS, and then add 200 μl of 0.5% paraformaldehyde. Labeled cells were scanned by FACS Calibur.

Virus replication and viral DNA quantitation

For viral replication, 1×10^5 cells per well were seeded in 6 well plates, infected with Ad at a multiplicity of infection (MOI) of 100 vp/cell in duplicate, and then incubated under normoxia or hypoxia. At the indicated time points, visual evidence of cytopathic effect (detachment) was noted, and the cells were collected. Virus was harvested from these samples by three freeze/thaw cycles followed by microcentrifugation for 5 min at 13,000 rpm. Adenovirus DNA was then extract using the Hirt methods (Hirt B., 1967) and quantified by Optical Density.

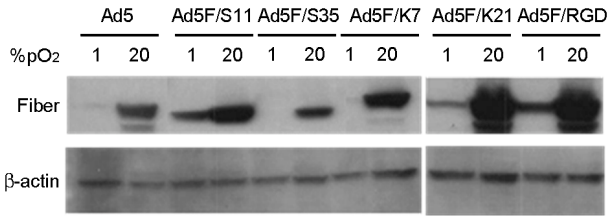

Fig. 1. Viral fiber protein levels in hypoxic and normoxic conditions. 293 cells were infected with 100 vp/cell. The cells were incubated in 1% or 20% oxygen for 3~4 days. Immunoblots for fiber and β-actin were performed. β-actin levels is used as an internal control.

Immunoblotting

Cell grown for 4 days under hypoxic or normoxic conditions were harvested and lysed in 5% SDS buffer. Total protein concentration was determined using the BCA protein assay (Pierce, Rockford, IL, USA), and equal amounts of protein were electrophoresed on a denaturing 4~20% polyacrylamidegel (Invitrogen). Proteins were transferred to a NC membrane (Bio-Rad, USA). Nonspecific binding was blocked in 5% milk in PBS-t (0.1% Tween-20). Antibodies were incubated in 5% skimmilk in PBS-t. The adenovirus fiber antibody Ab-4 (RDI, CA, USA) was used at a dilution of 1:400. The appropriate anti-mouse antibody (Santa Cruz) was used at a dilution of 1:5,000 for fiber detection. Antibody binding was visualized by enhanced chemiluminescence following the manufacturer's instructions (SuperSignal West Pico Chemiluminescent Substrate, Pierce, Rockford, IL, USA).

β-galactosidase (β-gal) assay

For adenoviral infections, tumor cells (5×10^5 to 1×10^6 cells) were seeded in 6 well plates. After tumor cells grow confluent, the media was changed 2 ml of Opti-MEM (Invitrogen Corporation Carlsbad, CA). After 1 h, cells were exposed to infected 1000 viral particles (vp) of vector per cells in 100 μl of Opti-MEM. Expression of the transgene was determined 48 hours after transduction. Some of the samples were analyzed using a β-gal enzyme assay system (Promega, Madison, WI), in this case, the cells were lyzed and processed as described by the manufacturer. The assay was performed by adding to the cell lysates an equal volume of Assay 2× Buffer that contains the substrate ONPG (*o*-nitrophenyl-β-D-galactopyranoside). Samples were then incubated for at 60 minutes, during which time the β-gal hydrolyzes the colorless substrate to *o*-nitrophenyl, which is yellow. The reaction was terminated by addition of sodium carbonate, and the absorbance was measured at 420 nm by spectrophotometry

Statistical analysis

Statistical significance was determined by standard *t*-testing. A p value of <0.05 was considered statistically significant.

RESULTS

Adenovirus replication and viral DNA quantitation in hypoxia

To compare the virus replication and viral DNA quantitation of fiber modified adenovirus, virus fiber was detected by immunoblotting and viral DNA was detected by Hirt method. In hypoxia, the formation of virus particle is significantly inhibited in all fiber modified adenovirus types (Fig. 1). And viral DNA synthesis also was inhibited about 30~50% in hypoxic condition compare with normoxic condition (Fig. 2A). But, gene expression levels in hypoxic condition are as same as in normoxic condition except Ad5F/K7 and Ad5F/K21 (Fig. 2B). These data show that oncolytic adenovirus replication and gene expression has different mechanism in hypoxic condition.

Gene expression efficiency of fiber-modified oncolytic adenovirus in hypoxic condition

To compare gene expression efficiency of each fiber modified vector, firstly, the cell surface receptor (CAR, CD46) expression in hypoxic conditions was monitored at three different tumor cell lines by FACS analysis. CAR expression is 98% in H1299 cells, 10~12% in PC3 cells, and 2~3% in MDA-MB-435 cells, but CD46 expression is above 90% in all three cell lines. However, there are no big difference of surface receptor expression between hypoxic and normoxic condition (Table 2).

There are no difference on gene expression between hypoxic and normoxic condition at H1299 cell lines (Fig.

3A) which has 98% CAR, 99% CD46 expression and PC-3 cell lines (Fig. 3B) which has 10% CAR and 98% CD46 expression. But, in case of MDA-MB-435 cell lines (CAR negative, CD46 positive), which has 3% CAR, 98% CD46 expression, gene expression of Ad5, Ad5F/S11, and Ad5F/S35 in hypoxic condition significantly was decreased compare to normoxic condition (Fig. 3C). And, in normoxic condition, gene expression of Ad5F/S11, S35 (CAR independent) is significantly increased compare to Ad5. And, in all three cell lines, gene expression of Ad5F/K7, K21, RGD is lower than that of Ad5, Ad5F/S11, and Ad5F/S35. These data show that gene expression of oncolytic adenovirus in hypoxic condition depends on cell surface receptor

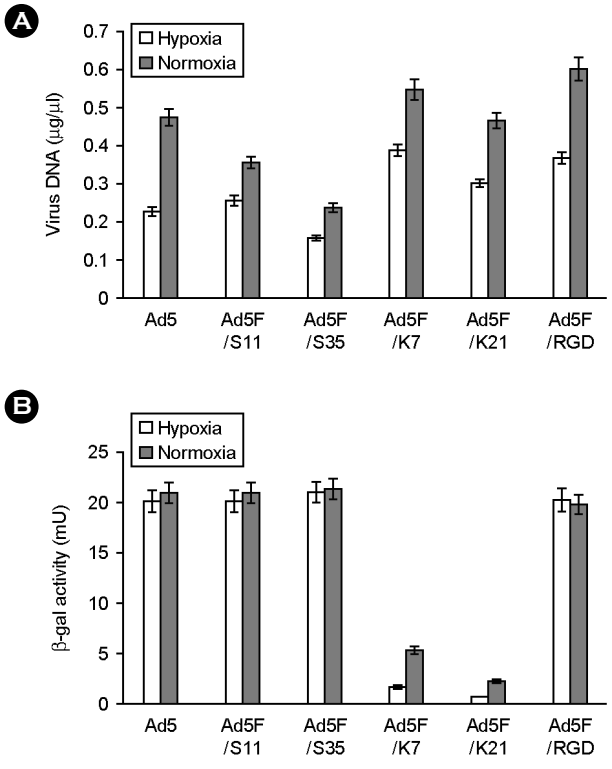

Fig. 2. Viral DNA quantitation and gene expression in hypoxic and normoxic conditions. 293 cells were infected with 100 vp/cell. The cells were incubated in 1% or 20% oxygen for 3~4 days for DNA quantitation (A) and for 48 h for gene expression (B). DNA quantitation was tested by Hirt method, and gene expression was examined by using β-gal assay kit.

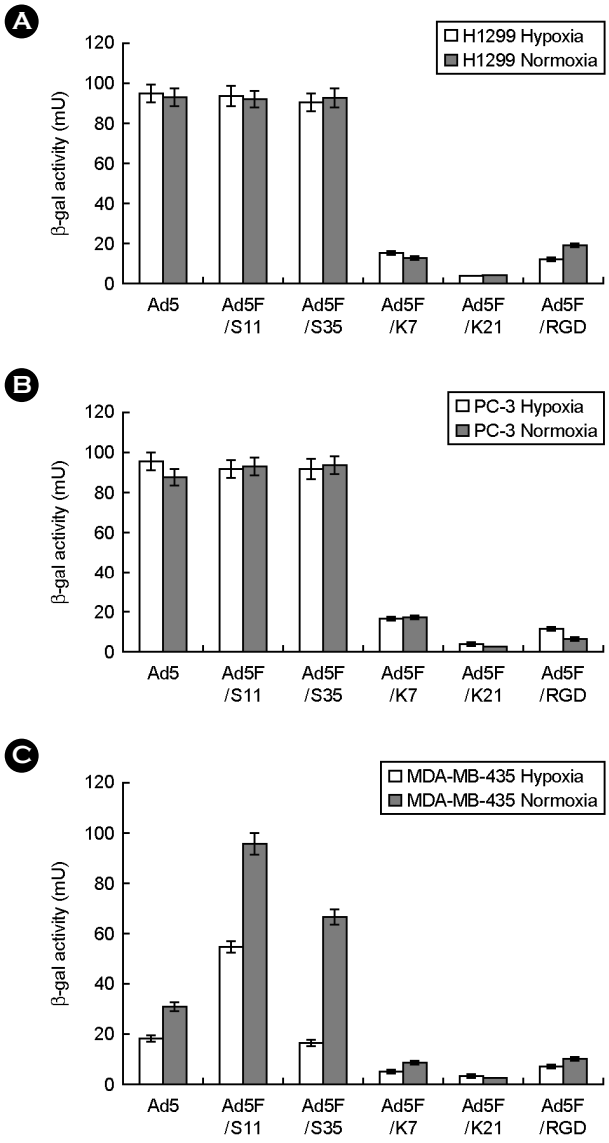

Fig. 3. Gene expression of adenoviral vector in hypoxic and normoxic conditions at various tumor cell lines. H1299 (A), PC-3 (B), MDA-MB-435 (C) cells were infected with 1000 vp/cell. The cells were incubated in 1% or 20% oxygen for 48 h. Gene expression levels were determined by β-gal assay kit.

Table 2. Cell surface receptor in hypoxic and normoxic condition

	H1299		PC3		MDA-MB-435	
	Hypoxia	Normoxia	Hypoxia	Normoxia	Hypoxia	Normoxia
CAR	98.88	98.09	10.37	12.94	2.08	3.39
CD46	99.35	99.7	97.59	98.94	92.69	98.05

H1299, PC-3, MDA-MB-435 cells were incubated in 1% or 2% oxygen for 48 h. Cell surface receptor expression levels were determined by FACS analysis.

(such as CAR and CD46) and tumor cell types.

DISCUSSION

The use of Ad5 for gene therapy for solid tumor has been poorly successful despite its great efficiency in preclinical studies, meanly because of its low transduction efficiency of tumor that express a very low level of CAR (Cripe et al., 2001; Kasono et al., 1999; Miller et al., 1998; Okegawa et al., 2001; Vanderkwaak et al., 1999). It has reported that down-regulation of CAR relate to tumor aggressiveness in bladder cancer (Okegawa et al., 2000). Numerous works have been done to improve oncolytic Ad5 efficiency for tumor treatment. These strategies related to the binding specificity, the length and the flexibility of the fiber that affect the interaction of the Ad with the targets. In this study, we proposed to identify the transduction efficiency of fiber modified oncolytic adenovirus in hypoxic condition. The optimal cancer therapy using oncolytic adenovirus relies on the efficient spread of virus. But, in solid tumor, hypoxia is the prevalent characteristics, and adenovirus primarily infects cells exposed to ambient oxygen concentrations (Pipiya et al., 2005).

Fig. 1 data show that all fiber modified oncolytic adenovirus (F/S35, F/S11, F/RGD, F/K 7 and 21) decrease the fiber protein expression that is needed for virus replication. Virus DNA replication itself decrease 30~50%, but their reporter gene expression is not changed (Fig. 2). These data show that infection efficiency of adenovirus is not changed between hypoxic and normoxic condition, but virus replication machinery that is essential spreading of oncolytic adenovirus is significantly affected in hypoxic condition. So various strategies is considered for the efficient cancer treatment of oncolytic adenovirus such as HIF-1α, transcription factor that is stable under hypoxia (Cho et al., 2004; Post et al., 2004). The gene expression on tumor cell lines is dependent on CAR expression. CAR positive and low cell lines (98% in H1299, 12% in PC-3) show almost same gene expression levels between hypoxic and normoxic condition. But, CAR negative cell lines (3% in MDA-MB-435) significantly decrease their gene expression in hypoxic condition, especially in case of Ad5, Ad5F/S11, S35 (Fig. 3). It has reported that the receptor of K21 (heparan sulfate) and RGD (αv integrins) are known to be expressed by a wide range of cells, as is the CD46 recently described to be Ad11 receptor (Segerman et al., 2003) and potentially Ad35 thus, Ad5F/S35 receptor. This broad expression of their receptor could explain the efficiency of the vector to transduce more diverse tissues than Ad5 does. But, in this study, K21 and RGD containing adenovirus has very low gene expression compare to Ad5, Ad5F/S11, 35 in both hypoxic and normoxic condition. Ad5F/S11 and S35 show higher or same gene expression in CAR negative or CAR positive cell lines, respectively. It has also reported that application with Ad35 was not impaired by a previous infection to Ad5 (Vogels et al., 2003). None of the patients in immunocompromised patients naturally infected by Ad35 showed neutralizing antibodies in their serum (Flomenberg et al., 1987). Ad5F/S11 and Ad5F/S35 which has CAR independent cellular tropism are best choice for tumor tissues of hypoxic region in our study.

In conclusion, these results suggest that the type of adenovirus must be strongly considered for efficient cancer treatment, especially is targeted for tumor which has hypoxic region.

Acknowledgements

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