SHORT COMMUNICATION

Bovine AAV transcytosis inhibition by tannic acid results in functional expression of CFTR in vitro and altered biodistribution in vivo

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Bovine adeno-associated virus (BAAV) can enter a cell either through a transcytosis or transduction pathway. We previously demonstrated that particles entering via the transcytosis pathway can be redirected to transduce the cell by blocking particle exocytosis with tannic acid (TA). To investigate whether this approach is useful in lung gene therapy applications, we tested the effect of TA on BAAV transduction in cystic fibrosis airway epithelia in vitro, and in mouse lung in vivo. Our findings suggest that BAAV transcytosis can occur in vitro and that treatment with TA reduces transcytosis and increases lung transduction. TA treatment did not impair the sorting and the activity of the BAAV expressed cystic fibrosis transmembrane regulator membrane protein.

RESULTS AND DISCUSSION

We first incubated primary cultures of human airway epithelia (HAE) with BAAV-green fluorescent protein at different concentrations of TA for 3 h. A total of 48 h post incubation, green fluorescent protein-positive cells were observed by fluorescent microscopy and quantified by determining the mean fluorescence intensity (Figures 1a and b). TA-mediated BAAV transduction was observed to be dose dependent with extensive transduction at a TA concentration of 0.125% w/v, a concentration fourfold lower than that reported previously.1

Cells treated with TA and no BAAV did not differ from those incubated with BAAV without TA (data not shown).

To determine whether TA-mediated BAAV transduction was also time-dependent, we incubated cells with 0.015% w/v TA for 3 or 24 h, then measured transduction at 48 or 96 h post incubation. At 48 h post incubation, we observed only a few positive cells (data not shown). However, by 96 h post incubation, a majority of the cells were positive (Figures 1c and d).

Taken together, these experiments demonstrate the effect of TA is both dose- and time-dependent and can increase transduction at concentrations much lower than that reported previously.1

For a number of pulmonary gene therapy applications, delivery via the apical surface of the lung may be the preferred route of vector delivery. As part of the innate immune system, however, a number of factors are secreted from the apical surface to prevent infection or colonization. Mucins have previously been shown to inhibit the transduction of some sialic acid binding AAV serotypes such as AAV4.2 To test the effect of mucin on TA-mediated BAAV transduction of HAE, primary cultures were prepared and either extensively washed to remove cell surface mucins, or left untreated before the addition of TA to the basolateral surface and BAAV vector encoding green fluorescent protein to the apical surface. Transduction was quantified at 48 and 96 h post vector addition (Figures 1e–g). At 48 h post incubation, transduction was reduced in the cultures containing mucin compared with the washed cultures (Figures 1e and g); however, this reduction was less evident by 96 h (Figures 1f and g). No transduction was detected in cells incubated with BAAV without TA. This experiment suggests that mucins slow TA-mediated BAAV transduction, but similar to AAV5, TA-mediated BAAV transduction is insensitive to mucins.

In vitro data on differentiated HAE suggests that utilizing the transcytosis entry pathway of BAAV can achieve significant transduction of HAE. For this to occur, BAAV particle trafficking must be altered by treatment with the transcytosis inhibitor TA. Although the

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INTRODUCTION

In AAV transcytosis studies, we used tannic acid (TA) to inhibit vector exocytosis from the basolateral side of polarized epithelial barrier models.1 As a consequence of viral transcytosis inhibition, the vector was redirected to the nucleus, resulting in a dramatic increase in transduction. To better understand the response of primary epithelial cells to TA treatment, as well as maximize transduction at the lowest concentration of TA, we performed a series of experiments varying TA concentrations and incubation times. Because TA treatment might result in disruption or damage to epithelial integrity, thus affecting bovine adeno-associated virus (BAAV) transcytosis, we monitored the transepithelial resistance as a parameter of epithelia integrity. No loss of transepithelial resistance was noted at the TA concentrations reported.

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In vitro data on differentiated HAE suggests that utilizing the transcytosis entry pathway of BAAV can achieve significant transduction of HAE. For this to occur, BAAV particle trafficking must be altered by treatment with the transcytosis inhibitor TA. Although the
exact mechanism of action of TA is not clear, it is reported to inhibit the membrane distribution of proteins that traffic from the basal side to the apical side.\textsuperscript{3,4} This effect could alter the trafficking of the recombinant proteins delivered by the BAAV vector. To test for an effect on genes delivered by BAAV vectors, we studied the biodistribution and activity of the cystic fibrosis transmembrane regulator (CFTR) gene delivered to cystic fibrosis (CF) HAE epithelia via BAAV vectors, following treatment of cultures with TA. We have previously demonstrated that a truncated form of this gene (CFTR\textsubscript{D}) can be delivered to CF HAE with an AAV5 vector, and that transduction resulted in an increase in cAMP-stimulated bumetanide-sensitive current.\textsuperscript{5} Immunocytochemical staining of the TA-treated and BAAV-CFTR\textsubscript{D}-transduced CF HAE suggested apical distribution of the CFTR\textsubscript{D} protein (Figures 2a–c), and was similar in distribution to that of CFTR\textsubscript{D} protein expressed by an adenovirus vector used as positive control, (Figures 2d–f) applied to the basolateral surface of the cells.\textsuperscript{6,7} To verify that the expressed CFTR\textsubscript{D} could correct the CF chloride transport defect, we applied BAAV-CFTR\textsubscript{D} for 4 h to the apical surface of differentiated primary cultures of CF HAE in the presence of TA. Seven days post incubation, we measured transepithelial chloride current and detected a CAMP-stimulated bumetanide-sensitive change in current in the TA BAAV-CFTR\textsubscript{D}-transduced cells (Figures 2g and h). The correction in the chloride current was dose dependent (1–8.5 \(\Delta\mu\text{A cm}^{-2}\)) and approached chloride current levels of our previous data for non-CF airways (8–15 \(\Delta\mu\text{A cm}^{-2}\)).\textsuperscript{5} A similar degree of current correction was also obtained with an adenovirus vector (Figure 2h).

As CF is a chronic condition, we next studied the persistence of CFTR\textsubscript{D} expression following BAAV TA-mediated transduction. Long-term observation of BAAV-CFTR\textsubscript{D} transduced cultures suggested a decrease in transduction activity over time. As shown in Figure 2h, by 21 days post transduction, the average current had decreased fourfold compared with the level of chloride current correction measured on day 7. Thus, activity may decline over time due to decreased protein expression, although TA-mediated BAAV transduction does not alter the distribution of CFTR\textsubscript{D} or protein function.

To determine whether TA-mediated BAAV transduction could occur \textit{in vivo}, TA was injected into mice by intraperitoneal injection, followed by delivery of BAAV vector encoding luciferase (Luc) to the lungs via intranasal administration. The level of transduction was observed and quantified by the Xenogen camera imaging system (Hopkinton, MA, USA) (Figures 3a and b). At 24–72 h post administration, little luminescence signal was detected in the lung of control or mice treated with 1 mg TA. However, pretreatment of mice with TA increased doses of TA (2 and 3 mg) enhanced BAAV transduction of the lungs two and eightfold, respectively, compared with controls (Figures 3a and b). \(N=2\) in duplicate. The Student’s \(t\)-test \(P\)-value < 0.05. Positive cells of four random fields in experiment A were also counted and similar fold changes compared with control were measured (data not shown).

Figure 1 Dose, time and mucins effects on primary HAE BAAV TA-mediated transduction. (a, b) Monolayer of differentiated HAE, plated in 6 mm transwell filters, were incubated apically with 10\(^8\) DNAse-resistant particles of BAAV-green fluorescent protein (GFP) and treated on the basolateral surface with 0, 0.031, 0.062 or 0.125, % w/v of TA for 3 h. At 48 h post incubation, GFP-positive cells were observed by fluorescent microscopy and fluorescence quantified using the ImageJ software (version 1.41o, Java 1.6.0_10, NIH, MD, USA; http://rsb.info.nih.gov/ij/download.html). (c, d) As above, cells were incubated apically with BAAV-GFP but treated with 0.015% w/v of TA for 3 or 24 h, respectively. After 96 h, GFP-positive cells were observed and quantified. (e–g) HAE cultures secreting mucins were extensively washed with cell medium or left untreated. BAAV was applied apically and cell treated on the basolateral surface with 0.25 or 0.5% w/v TA for 3 h. After 48 (e) and 96 (f) h positive cell were observed and quantified. \(N=2\) in duplicate. The Student’s \(t\)-test \(P\)-value < 0.05. Positive cells of four random fields in experiment A were also counted and similar fold changes compared with control were measured (data not shown).
Figure 2 Immunostaining and current tracing of TA BAAV-CFTRΔR-transduced CF HAE. Immunostaining of differentiated airway epithelia expressing BAAV-CFTRΔR (10^6 genomes (g.c.)/cell) (panels a–c) or ad5-CFTRΔR (100MOI) (d–f). Data are X–Y confocal images (b, c and e, f) or en face images (a and d). Cftr is shown in green, tight junction protein zonula occludens-1 in red and indicated by arrows of the same color, respectively. 46-Diamidino-2-phenyl indole nuclear stain is in blue. (g) Current tracings of CF airway epithelia transduced with the indicated amounts of BAAV-173CMV-CFTRΔR (10^6 or 10^4 g.c./cell). Millicells were treated sequentially with, amiloride (10^-4 M), forskolin (10^-4 M)/IBMX (10^-5 M) and bumetanide (10^-4 M) as indicated. (h) Bumetanide-sensitive forskolin-stimulated current (in μamps cm^-2) in well-differentiated CF epithelia expressing the indicated amount of BAAV-CFTRΔR at 7 or 21 days post transduction. N=3 for each AAV viral titer; N=1 for ad5-CFTR (4 days post-infection) studied on the 7th and 21st day after BAAV infections. One-way analysis of variance with Bonferroni’s post test *P-value <0.05.
had no effect on AAV2 transduction, which is not reported to have transcytosis activity in HAE (Figure 3c).

Recent work has demonstrated that chitotriose is an important attachment molecule in BAAV transcytosis. To test if this carbohydrate is also important in vivo in TA-mediated transduction, BAAV vector was incubated with chitotriose before lung instillation in TA-treated mice. As previously reported in vitro, preincubation of BAAV vector with chitotriose blocked TA-mediated transduction in vivo (Figure 3c, TA versus TA + chitotriose). Immunofluorescent staining of sections from the TA-treated BAAV-infected mice showed transduction of alveolar cells (Figure 3d).

To determine whether TA-mediated BAAV transduction persisted in vivo, mice were treated with or without TA intraperitoneal followed by intranasal delivery of BAAV encoding Luc and expression was measured 24 h or 8 weeks post delivery (N=4/group). In isolated organs at 24 h post delivery, mice treated with BAAV alone showed some transduction in the lung and stomach (Figures 4a–c). BAAV+TA-treated mice showed an increase in Luc activity in the lung, whereas that of the stomach decreased compared with the BAAV alone treated mice (Figures 4a and c). At 8 weeks post delivery, the level of Luc in BAAV+TA-treated animals was similar to the BAAV alone treated mice (Figure 4b). The stomachs of the BAAV+TA-treated mice still maintained a lower level of activity compared with BAAV alone treated mice; however, the decrease was not statistically significant (Figure 4c).

Just as the use of alternative serotypes of vectors has enhanced gene transfer to the lung, the addition of chemical modulators of vector trafficking could also enhance transduction. TA induces re-trafficking of BAAV vectors, which can markedly increase BAAV transduction activity without affecting localization or activity of the therapeutic proteins encoded by the vector. Although TA treatment does not alter the overall long-term transduction of the lung by BAAV in vivo, it does increase the kinetics of transduction. Following intranasal delivery of BAAV vector, transduction could be detected in other tissue, such as the stomach and the liver. This finding supports our previous in vitro observations of transcytosis activity with BAAV vectors. Transcytosis of vector can be inhibited by treatment with TA in vivo, which then results in enhanced transduction at the site of delivery and consequent limited biodistribution.

An interesting observation in our study is the rapid kinetics of BAAV transduction induced by TA treatment compared with other permissive AAVs in the lung. Previous research has demonstrated that other factors, such as addition of a helper virus, can markedly increase the time course of transduction. Although the complete mechanism for helper virus-assisted transduction is not clear, several steps such as trafficking to the nucleus, and second strand synthesis are likely affected. Although there is no research addressing TA involvement in cellular DNA second strand synthesis or in nuclear transport, our research suggests these pathways are likely affected by TA.

TA is a complex mixture of compounds contained in many plants that are consumed by humans. In addition, it has been used medically to treat burns and some of its biological properties are known at the molecular level. As it is currently available, high concentrations of TA will affect transepithelial resistance of cells grown in transwells, limiting its use. However, fractionation may allow the isolation of the...
active compounds affecting BAAV transcytosis and increase TA’s therapeutic window for in vivo use.

In this manuscript we have established a proof of concept that blocking the transcytosis pathway of BAAV markedly increases both the kinetics and overall transduction activity.

CONFLICT OF INTEREST
The authors declare no conflict of interest.

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