Transplant cytochalasin-D treatment induces apically administered rAAV2 across tight junctions for transduction of enterocytes

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Enteropathogens are known to disrupt apical actin filaments and/or tight-junction barriers of intestinal epithelial cells to promote infection. In this study, we show that a controlled, cytochalasin-D (Cyto-D)-mediated disruption of actin filaments and tight junctions enhanced the apical delivery of the gene-therapy vector recombinant adeno-associated virus serotype 2 (rAAV2). This increase in transduction efficiency can be attributed to the enhanced delivery of rAAV2 across the Cyto-D disrupted tight junctions, allowing basolateral entry of rAAV2.

Previously, we have shown that MG101 and doxorubicin are capable of overcoming proteasome-mediated transduction barriers of rAAV2 in enterocytes. In this study, when Cyto-D was combined with MG101 and doxorubicin in apical delivery of rAAV2 to transduce the differentiated Caco-2 enterocytes, a synergistic >2300-fold increase in transgene expression was achieved. We conclude that Cyto-D is capable of permeating the polarized enterocytes for rAAV2 transduction, which may potentially be a useful device to facilitate intestinal gene transfer via the gut lumen.

Luminal delivery of gene-transfer vectors by oral formulations or endoscopic procedures provides a non-invasive approach to genetically modify gut mucosa by either transgene expression or gene silencing. Gene therapy offers significant promise for treating nutrient transporter deficiencies or inflammatory bowel disease (Prieto et al., 2003; Wirtz & Neurath, 2003; Tang & Sibley, 2006). Furthermore, the relative ease of vector administration to the gut makes it an attractive potential target for the treatment of non-intestinal diseases, e.g. diabetes (Cheung et al., 2000), and even for oral vaccines (During et al., 2000).

Among the available gene-transfer methods, the recombinant adeno-associated virus serotype 2, rAAV2, based vectors continue to attract interest. Promising results in clinical trials of targeting gene delivery to the liver (haemophilia B) and the lung epithelial tissues (cystic fibrosis) provide evidence that rAAV2-mediated gene therapy maybe effective (Moss et al., 2004; Manno et al., 2006). Despite significant progress using rAAV2 as a therapeutic gene carrier, studies targeting rAAV2 delivery to the gut lumen have been associated with low levels of rAAV2 transduction of intestinal epithelial cells. Specifically, delivery of rAAV2 through oral gavage to the rat small intestine resulted in the transduction of primarily non-epithelial cells in the lamina propria (During et al., 1998). Additionally, Shao et al. (2006) were unable to detect in vivo transduction of the mouse gastrointestinal tract with a vector encoding the β-galactosidase reporter. These findings suggest that intestinal epithelial cells possess barriers that prevent rAAV2 attachment, internalization and/or intracellular trafficking of rAAV2 from the apical route, thereby resulting in limited transduction efficiency.

Previously, we have revealed proteasome-mediated barriers to rAAV2 transduction in intestinal epithelial cells (Tang et al., 2005). Delivery of rAAV2 vectors to polarized human Caco-2 enterocytes in transwells resulted in preferential transduction at the basolateral surface following treatment that could be enhanced by treatment with the proteasome-modulating agents MG101 and doxorubicin. The low levels of transduction via the apical surface suggested that increasing the permeability of the gut epithelium to provide for basolateral exposure could facilitate rAAV2 intestinal gene transfer from the luminal route. The gastrointestinal tract has natural barriers that play an important role in protecting against microbial infections. These microbial defences also counter strategies to target gene delivery to the gut mucosa. Enteropathogens have evolved to overcome these defence mechanisms and breach the infection barriers. Certain pathogens are known to attack the microvillar actin and/or tight junctions to penetrate the layer of intestinal epithelial cells (Sousa et al., 2005). Importantly, studies of actin rearrangement induced by micro-organisms have identified a spectrum of

Supplementary figures are available with the online version of this paper.
chemicals that target actin polymerization (Spector et al., 1999). Despite the feasibility of using these actin-disrupting chemicals to manipulate the actin network and the tight-junction permeability, to our knowledge, this strategy has not been used previously to improve the permissiveness of gut mucosa to gene-transfer vectors.

In this study, we sought to investigate the effect of transient cytochalasin-D (Cyto-D)-mediated disruption of actin filaments and tight junctions on the permissiveness of polarized intestinal epithelial cells to rAAV2. Cyto-D is a cell-permeable fungal toxin and a potent inhibitor of actin polymerization, which binds to the plus end of the polymeric fibrous actin and prevents the addition of monomeric globular actin (Sampath & Pollard, 1991). Because actin filaments associate with the tight-junction proteins such as ZO-1, the Cyto-D-mediated actin disintegration compromises the tight-junction seal and generates a ‘leaky’ epithelium (Wells et al., 1998). In this study, results using Cyto-D alone or combined with proteasome-modulating agents to induce apically administered rAAV2 across tight junctions for transduction of intestinal epithelial cells in culture are presented.

To assess the kinetic effects of Cyto-D (Sigma) on polarized human Caco-2 enterocytes (>2 weeks of cultivation), a voltohmmeter (World Precision Instruments) was used to measure the trans-epithelial electrical resistance (TEER) of cell monolayers, the readout being a measure of tight-junction permeability (Fig. 1a). Cyto-D was added from a stock solution (2 mg ml\(^{-1}\) in DMSO) for 4 h. Previously, Wells et al. (1998) showed that 1 μg Cyto-D ml\(^{-1}\) was more effective in decreasing the TEER of mature Caco-2 cultures than 0.1 or 10 μg ml\(^{-1}\). Stevenson & Begg (1994) also observed a concentration-dependent effect of Cyto-D on tight junctions in MDCK epithelial cells with a higher potency at 2 rather than 20 μg ml\(^{-1}\) in decreasing the TEER. We adopted a similar test range and found that Cyto-D at concentrations of 1 and 10 μg ml\(^{-1}\) lowered TEER to approximately 54 and 40 %, respectively, of that at time 0 over a period of 4 h. Upon withdrawing Cyto-D, the TEER gradually returned to initial levels. Cells treated with 1 and 10 μg Cyto-D ml\(^{-1}\) took approximately 4 and 8 h, respectively, to restore the TEER level to that of untreated cells.

Although Cyto-D has been widely used as an actin-disrupting agent, its toxicity is not well-characterized. We next sought to evaluate the cytotoxicity of Cyto-D on polarized Caco-2 cells. The mitochondrial activity (MTS assay; Promega) in the treated cells after 4 h of Cyto-D (1 μg ml\(^{-1}\)) treatment was only slightly decreased (11 %). Upon withdrawing Cyto-D, the mitochondrial activity recovered to that of untreated cells (Supplementary Fig. S1 available in JGV Online). These results indicated that the treatment exhibited minimal metabolic toxicity toward the differentiated Caco-2 cells during the test period.

To visualize the effect of Cyto-D directly, actin filaments and DNA were stained with tetramethyl rhodamine isothiocyanate (TRITC)-conjugated phalloidin and 4,6-diamidino-2-phenylindole (DAPI; Chemicon), to reveal the transient disruption of actin filaments when viewed using a confocal microscope (LSM510; Carl Zeiss). The actin network at 4 h showed disintegration of the cortical actin network beneath the lateral plasma membrane and formed punctate actin foci, and by 8 h after withdrawing Cyto-D, the actin organization was restored (Fig. 1b–d). Because actin filaments directly link to the tight-junction proteins, the same treatment also disrupted the tight-junction connections as revealed in immunofluorescence experiments using a mAb to ZO-1 protein (Invitrogen).
(Fig. 1e–g). The effects on actin filaments and tight junctions were consistent with the TEER data (Fig. 1a): the effects of the 4 h Cyto-D treatment of the polarized Caco-2 enterocytes are transient and reversible.

We next assessed the effect of Cyto-D treatment on the transfer of rAAV2 particles (genome copies, TaqMan PCR method described in the Supplementary data, available in JGV Online; Applied Biosystems) (Veldwijk et al., 2002) across polarized Caco-2 monolayer. When rAAV2 (3×10^8) was added to the upper (donor) compartment of the transwell, approximately 9×10^7 copies were found in the lower (recipient) compartment, while 1.8×10^8 copies remained in the upper compartment and 1.2×10^8 copies were associated with the Caco-2 monolayer (Fig. 2a). On the other hand, when Cyto-D was not used in the test, the rAAV2 particles were undetectable in the lower compartment. No significant change was found in the amount of vector associated with Caco-2 cells by the Cyto-D treatment. A luciferase assay (Promega) was used to quantify rAAV2-luciferase–enhanced green fluorescent protein (EGFP) (Tang & Sambanis, 2003) transduction of polarized intestinal epithelial cells in culture. Treatment with 1 and 10 μg Cyto-D ml^-1 resulted in 8.0 (±1.0)-fold and 6.6 (±3.0)-fold inductions of apical rAAV2 delivery, respectively (Fig. 2b). In contrast, the delivery was not affected by the Cyto-D treatment when the rAAV2 exposure was from the basolateral domain of the polarized Caco-2 enterocytes in transwells. Additionally, apical rAAV2 delivery to differentiated T84 enterocytes (>3 weeks of cultivation, TEER ~ 1000 ohm cm^-2) in the presence of Cyto-D resulted in 8.4 (±3.3)-fold and 1.3 (±0.5)-fold inductions when the concentrations of Cyto-D were 1 and 10 μg ml^-1, respectively. These results indicate that transient actin/tight junction disruption, mediated by Cyto-D treatment, enhances apical rAAV2 delivery to transduce differentiated enterocytes. It is noteworthy that 1 μg Cyto-D ml^-1 was more potent than 10 μg Cyto-D ml^-1 in achieving the induction.

Four additional epithelial cell lines (HT-1080 fibroblasts, 293 cells, HeLa cervical carcinoma cells and HepG2 hepatic cells) were examined for their permissiveness to rAAV2 transduction upon treatment with Cyto-D. Unlike Caco-2 cells, all four cell lines tested exhibited a reduction in rAAV2 transduction in response to Cyto-D treatment. Specifically, Cyto-D (1 μg ml^-1) resulted in 75, 52, 60 and 70 % decreases in transduction efficiency in HT-1080, 293, HeLa and HepG2 cells, respectively (Supplementary Fig. S2 available in JGV Online). Since Cyto-D disrupts the host cell cytoskeleton and in turn inhibits clathrin-dependent endocytosis (Carreno et al., 2004), the opposite effects of Cyto-D on transducing the polarized Caco-2 enterocytes and the non-polarized epithelial cells suggest distinct barriers to rAAV2 exist for different cells.

Previously, we have shown that the proteasome-modulating agents MG101 and doxorubicin are capable of overcoming proteasome-mediated transduction barriers to rAAV2 in enterocytes (Tang et al., 2005). Cyto-D (1 μg ml^-1) was therefore combined with MG101 (400 μM) and doxorubicin (5 μM) in apical delivery and following transduction the differentiated Caco-2 enterocytes exhibited a >2300-fold increase in transgene expression compared with 8- and 37-fold inductions by Cyto-D alone and proteasome inhibitors (MG101 and doxorubicin) alone, respectively (Fig. 3a). Representative fluorescence micrographs illustrate EGFP expression in the Cyto-D+MG101+doxorubicin-treated Caco-2 monolayers transduced at three different levels of m.o.i. (Fig. 3b–d).
Cyto-D induces apical rAAV2 delivery to enterocytes

Transduction barriers have been identified at different stages of rAAV2–host cell interaction; these stages include internalization, endocytosis, ubiquitination–proteasome pathway, uncoating and second-strand DNA synthesis (Tang et al., 2005; Duan et al., 2000; Thomas et al., 2004; Ferrari et al., 1996). Our results suggest that apical rAAV2 transduction of the polarized intestinal epithelium is limited in part by microvilli and tight junctions. Microvilli are responsible for luminal nutrient adsorption and are in constant contact with enteric microbes. To avoid microbial infections, it is reasonable to conceive that the luminal brush border microvilli would present a barrier to rAAV2 entry and thus prevent transgene delivery.

Recent studies have revealed that the rotavirus spike protein VP4 disruption of actin filaments for promoter infection: Cyto-D has a similar effect at a rate comparable with that observed in rotavirus infection (Gardet et al., 2006). Treatment with Cyto-D increases the internalization of bacteria Salmonella typhimurium and Proteus mirabilis to the polarized HT-29 and Caco-2 enterocytes (Wells et al., 1998). On the other hand, Cyto-D-induced actin disruption has also been associated with reduced infectivity of West Nile virus in African green monkey kidney epithelial cells (Chu & Ng, 2002, 2004), porcine circovirus 2 in porcine monocyte cell line 3D4/31 (Misinzo et al., 2005), and human polyomavirus JC virus in human glial cells (Ashok & Atwood, 2003). In this study, a similar reduction in rAAV2 transduction of 293, HT-1080, HeLa and HepG2 cells was observed. The reduced transduction efficiency maybe attributed to the interference of Cyto-D blocking the clathrin-dependent rAAV2 endocytosis. Actin dynamics are coupled to clathrin-coated vesicle formation at the trans-Golgi network (Carreno et al., 2004), and rAAV2 particles are typically transported to, and accumulated in, the Golgi apparatus after internalization (Pajusola et al., 2002). The mixed effects of Cyto-D on rAAV2 transduction efficiency may reflect diversity in the rAAV2–host cell interaction in different tissues.

It would be interesting to investigate the optimal administration concentration, stability and safety issues involved in using Cyto-D in vivo. In animal models, luminal administration of Cyto-D and rAAV2 can be achieved via oral gavage. Nonetheless, the present study constitutes an important step toward systematic understanding of rAAV2–intestinal cell interactions to facilitate gene transfer to somatic gut mucosa for potential therapeutic transgene delivery. Barriers to apical rAAV2 transduction have hindered the administration of rAAV2 from the gut lumen to target intestinal cells. However, we have demonstrated that transient treatment with Cyto-D can permeate tight junctions for basolateral rAAV2 exposure. Future experiments will be aimed at combining Cyto-D with other induction agents such as MG101 and doxorubicin to establish rAAV2-mediated intestinal gene transfer from the luminal route in vivo.

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References


Fig. 3. Synergistic induction of apical rAAV2 delivery in transducing the differentiated Caco-2 enterocytes treated with Cyto-D and proteasome-modulating agents. (a) Transgene expression in cells treated with Cyto-D alone, a mixture of proteasome inhibitors (MG101 and doxorubicin (Dox)), or combined Cyto-D and proteasome inhibitors. Experiments were performed in triplicate, except that six samples were used in the analysis of the combined treatment of Cyto-D and proteasome inhibitors. Bars indicate standard deviation. (b, c and d) Micrographs of rAAV2 transduction of differentiated Caco-2 enterocytes with m.o.i. at 10, 100 and 1000 genome copies (G.C.) per cell, respectively. Cells in parallel cultures were analyzed for reporter luciferase and EGFP expression 3 and 4 days after transduction, respectively.


