Specific Modulation of Airway Epithelial Tight Junctions
by Apical Application of an Occludin Peptide

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Abbreviations:
WD HAE: well-differentiated human airway epithelia
R:T: transepithelial resistance
ABSTRACT
Tight junctions are directly involved in regulating the passage of ions, and macromolecules (gate functions) in epithelial and endothelial cells. The modulation of these gate functions to transiently regulate the paracellular permeability of large solutes and ions could increase the delivery of pharmacological agents or gene transfer vectors. To reduce the inflammatory responses caused by tight junction-regulating agents, alternative strategies directly targeting specific tight junction proteins could prove to be less toxic to airway epithelia. The apical delivery of peptides corresponding to the first extracellular loop of occludin to transiently modulate apical paracellular flux has been demonstrated in intestinal epithelia. We hypothesized that apical application of these occludin peptides could similarly modulate tight junction permeability in airway epithelia. Thus, we investigated the effects of apically applied occludin peptide on the paracellular permeability of molecular tracers and viral vectors in well-differentiated human airway epithelial cells. The effects of occludin peptide on cellular toxicity, tight junction protein expression and localization, and membrane integrity were also assessed. Our data showed that apically applied occludin peptide significantly reduced transepithelial resistance in airway epithelia, and altered tight junction permeability in a concentration dependent manner. These alterations enhanced the paracellular flux of dextrans as well as gene transfer vectors. The occludin peptide redistributed occludin, but did not alter the expression or distribution of ZO-1, claudin-1 or claudin-4. These data suggest that specific targeting of occludin could be a better-suited alternative strategy for tight junction modulation in airway epithelial cells compared to current tight junction modulating agents.
Tight junctions are present at the apical ends of lateral membrane surfaces of epithelial and endothelial cells and form a series of discrete sites of apparent membrane fusion involving the outer leaflet of the plasma membranes of adjacent cells. Two main functions have been attributed to the tight junction, gate functions that regulate the passage of ions and macromolecules through the paracellular pathway, and fence functions that separate the apical and basolateral membrane domains of polarized epithelia and endothelia, and prevent the intermixing of membrane-domain proteins and lipids between the apical and the lateral membranes (Gumbiner, 1987; Gumbiner et al., 1991). Tight junctions comprise transmembrane proteins, such as occludin (Furuse et al., 1998), claudins (Furuse et al., 1998; Morita et al., 1999), JAM (Martin-Padura et al., 1998) and CAR (Cohen et al., 2001), as well as cytoplasmic molecules, such as ZO-1 (Stevenson and Goodenough, 1984), ZO-2 (Itoh et al., 1999), ZO-3 (Haskins et al., 1998), cingulin (Citi and Cordenonsi, 1998), and 7H6 (Zhong et al., 1993). Regulatory molecules including tyrosine kinases, proteases and GTPases also colocalize near the tight junction. Interactions between the transmembrane components and cytoplasmic molecules, along with the cytoskeleton and regulatory molecules, are thought to modulate the gate and fence functions of tight junctions.

Several groups have investigated the modulation of tight junction gate function as a method to enhance drug uptake in intestinal epithelia (van Hoogdalem et al., 1990; Swenson et al., 1994; Yamamoto et al., 1996). Like intestinal epithelia, the airway epithelium is also resistant to the uptake of apically delivered macromolecules. Tight junction-modulating agents such as EGTA, sodium caprate, the sodium salt of the saturated medium-chain fatty acid capric acid (C10) or lauric acid (C12), polidocanol and
lysophosphatidyl choline have been shown to increase the permeability of airway tight
junction and also enhance gene transfer (Parsons et al., 1998; Duan et al., 1998; Wang et
al., 2000; Coyne et al., 2000; Chu et al., 2001; Limberis et al., 2002; Coyne et al., 2003).
However, delivery of these agents that alter multiple proteins in the tight junction has
been linked to inflammation in airways in vitro and in vivo. Alternative strategies that
target specific tight junction proteins could prove to be less toxic to airway epithelia.
One such strategy directly targeted occludin to enhance tight junction permeability to
molecular tracers in a Xenopus kidney epithelial cell line A6, by basolateral delivery of
synthetic peptides corresponding to the second extracellular loop of occludin (Wong and
Gumbiner, 1997). A similar modulation in tight junction permeability of solutes in
intestinal epithelia by apical delivery of a synthetic occludin peptide has also been
reported (Tavelin et al., 2003). Tavelin et al. showed that the conjugation of a lipoamino
acid to the occludin peptide inhibited enzymatic degradation of the peptide by apical
peptidases. Suppression of occludin by stable expression of short interfering RNA with
associated changes in the gate functions of tight junctions in MDCK cells (Yu et al.,
2005) provides further evidence for the role of occludin in tight junction functions.

Thus occludin is a potentially good target for modulating tight junction barrier
function. Occludin is a ~60 kDa integral membrane protein of tight junction fibrils that
spans the membrane four times with three cytoplasmic domains and two extracellular
loops (ECLs). The first ECL has a high tyrosine and glycine composition, while the
second loop is rich in tyrosine residues. Both extracellular loops of occludin consist
solely of uncharged residues with the exception of one or two charged residues adjacent
to the membrane. Occludin localizes to tight junctions and its overexpression is known
to increase transepithelial resistance in mammalian epithelial cells (McCarthy et al., 1996). Alteration of occludin expression has been shown to increase epithelial permeability, and the absence of occludin from tight junctions has been shown to have no significant affect on tight junction morphology (Balda et al., 1996; Saitou et al., 2000).

We hypothesized that apically applied occludin peptide could specifically modulate tight junction permeability in airway epithelia, and enhance the paracellular flux of molecular tracers and viral vectors. To test this hypothesis, we addressed the following issues: (1) whether specific targeting of occludin in airway epithelial tight junctions by apical application of occludin peptide could enhance paracellular permeability to macromolecules such as dextrans and gene transfer vectors, (2) whether the specificity of occludin peptide would affect the expression and distribution of other tight junction proteins and (3) whether specific targeting of occludin could reduce the toxicity typically observed with other tight junction modulating agents.
MATERIALS AND METHODS

Occludin Peptide synthesis. The occludin peptide OP90-103 (Figure 1), corresponding to the first extracellular loop of human occludin and consisting of amino acids 90 to 103 (DRGYGTSLLGGSVG) was synthesized by a stepwise solid-phase procedure as previously described (Tavelin et al., 2003). Since a prior study showed that the conjugation of a lipoamino acid to the occludin peptide inhibited enzymatic degradation of the peptide (Tavelin 2003) by apical peptidases, OP90-103 was conjugated to a lipophilic amino acid moiety at the N terminus of OP90-103, resulting in C14-OP90-103. A scrambled peptide conjugated to the same lipophilic amino acid moiety (C14-SGLSGGTRDYGTGL-NH2) was used as a control in all experiments.

Cell Culture. Primary airway cells from human subjects were isolated in accordance with guidelines approved by the Committee on the Protection of the Rights of Human Subjects. Well-differentiated human airway epithelial (WD HAE) cells were isolated from surgical specimens, plated at a density of 2 × 10^5 cells/12 mm on Transwell-Col inserts (0.4-µm pore size), and maintained in a 50:50 mixture of LHC basal medium (Biofluids, Rockville, MD) and Dulbecco's modified Eagle's medium with 4.5g/L glucose supplemented with growth factors, retinoic acid, and bovine serum albumin as described previously (Fulcher et al., 2005). Upon reaching confluence, culture medium was aspirated from the apical surface and cells maintained at an air-liquid interface for 3 to 4 weeks. Cultures with >10% cilia as determined by microscopy, and a transepithelial electrical resistance (R_T) of ≥ 500Ω-cm² were selected for experiments.

Electrophysiological Measurements. The R_T of primary HAE cells was monitored with an ohmmeter (EVOM; World Precision Instruments, Sarasota, FL). Culture medium or
HEPES- Ringer solution (HBR) was added to the apical and basolateral surfaces of WD HAE cells in Transwell-Col inserts, and incubated for 20 min at 37°C following which $R_T$ was measured.

**Measurement of permeability.** To determine the optimal concentration of occludin peptide for increased tight junction permeability, a dose response curve was performed. Primary HAE cells were grown on Transwell-Col inserts under air liquid interface conditions as previously described (Fulcher et al., 2005). Occludin peptides were applied to the apical surface of 25-28 day old WD HAE cultures in the following concentrations: 10, 30, 100, 300, and 1000 $\mu$M. Control cultures were treated with vehicle (no peptide) or 1000 $\mu$M of the scrambled peptide. Transepithelial resistance ($R_T$) was monitored with an ohmmeter at 10 min. intervals. When $R_T$ decreased, occludin peptide was removed from the culture medium and the recovery of $R_T$ was monitored at 6 h, 24 h, and 48 h. The optimal concentrations of occludin peptide (300 $\mu$M and 1000 $\mu$M) that reduced $R_T$ rapidly (< 30 min) and allowed for full recovery of $R_T$ following occludin peptide removal, were selected for subsequent experiments. Once $R_T$ had decreased, the maximal effect of occludin peptide on the permeation of dextrans in WD HAE cells was measured. FITC-labeled dextrans of 70 kDa or 2000 kDa were applied at a concentration of 5 mg/ml to the lumen (source) following removal of the occludin peptide from the culture medium. The appearance of dextrans in the basolateral bath (sink) was measured in 10 $\mu$l samples obtained from the sink every 10 min over a 60 min period, and in 10 $\mu$l of source samples at time 0 and at 60 min. Fluorescence was measured in samples at 496 nm. The paracellular permeability ($P_{app}$) coefficients were calculated as previously described (Stutts et al., 1981).
Measurement of transduction efficiency. A recombinant, first generation, E1, E3 deleted adenovirus serotype 5 vector encoding a LacZ transgene (AdLacZ), and an adeno-associated vector encoding a green fluorescent protein (GFP) transgene (AAV2 U1a GFP) were prepared by the University of North Carolina at Chapel Hill Gene Therapy Vector Core. Cultures of WD HAE epithelia were apically exposed to 1000 µM scrambled peptide and 300 or 1000 µM occludin peptide for 20 min. AdlacZ, at a multiplicity of infection of 300, or AAV2 U1a GFP at 500 transducing units/cell, were applied to the lumen following removal of the occludin peptide from the culture medium. After infection for 2 hr at 37°C, cells were washed with PBS and incubated for an additional 48 hr for Ad-mediated LacZ detection. Adeno-associated vector-mediated GFP detection was performed by fluorescent microscopy at 4 weeks post transduction, which is the optimal time required for synthesis of the complementary strand in AAV expression. LacZ expression was detected by X-gal histochemistry. The cultures were stained in 5-Bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) for 4 hr at 37°C. To minimize background staining, the pH of all solutions was adjusted to 8.0 with Tris buffer (20 mM final concentration). LacZ protein levels were quantitated by β-Galactosidase enzymatic analysis (Galactostar Light assay, Tropix, Bedford, MA) according to the manufacturer’s instructions. GFP expression was detected by fluorescent microscopy. Vehicle treated cultures were not exposed to scrambled peptide or occludin peptide, but received the same concentration of viral vectors as the other treatment groups.

Measurement of cellular toxicity. Occludin peptide-induced cellular toxicity was assessed by the amount of lactate dehydrogenase (LDH) leakage into the culture medium,
since an increase in the number of cell-membrane damaged cells results in increased
LDH levels in the culture supernatant. WD HAE cells were apically exposed to 1000 µM
scrambled peptide or 300 µM and 1000 µM concentrations of occludin peptide. At 0, 6
and 24 h post treatment, culture medium was collected and LDH levels were measured
using a commercial kit [LDH release Detection Kit (LDH), Roche], and analyzed
according to the manufacturer's instructions. Briefly, 100 µl of cell-free supernatant was
added in duplicate to wells in a 96-well microtiter plate followed by the addition of 100
µl of LDH assay reaction mixture. After a 90 min incubation at room temperature, the
absorbance was read on an ELISA microplate reader at 492 nm. Background values were
subtracted from each reading, and the average absorbance for each sample was
calculated. The mean percent occludin peptide-induced LDH release for each sample
was calculated as follows:

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\text{% Occludin peptide-induced LDH release} = \frac{\text{ABS}_{\text{expt}} - \text{ABS}_{\text{low}}}{\text{ABS}_{\text{high}} - \text{ABS}_{\text{low}}} \times 100
\]

where \( \text{ABS}_{\text{expt}} \) = mean absorbance of treated cells
\( \text{ABS}_{\text{low}} \) = mean absorbance of culture medium
\( \text{ABS}_{\text{high}} \) = mean absorbance of Triton X-100 treated cells

**Transepithelial Permeability.** For visualization of paracellular permeability in live WD
HAE cells, cultures were treated with 300 or 1000 µM occludin peptide in HEPES-
buffered Ringer (HBR) containing 2 mg/ml Texas Red-labeled 70 kDa dextran, and XZ-
axis scans recorded by confocal microscopy at 1, 5, 10, 15, 20 and 30 min following
apical application.
Western Blotting. Well-differentiated human airway epithelial (WD HAE) cultures were apically exposed to 1000 µM scrambled peptide or 300µM and 1000µM occludin peptide. At 6 and 24 h post-treatment, whole cell lysates from occludin peptide-treated and control cultures were prepared with 0.1% Triton X-100 extraction buffer containing phenylmethanesulfonyl fluoride (PMSF) and dithiothreitol (DTT). Equal amounts of protein (50 µg) were loaded onto 12 % Tris- glycine gels (Novex, San Diego, CA). After electrophoresis for 1.5 h at 150V, protein was transferred to polyvinylidine difluoride (PVDF) membrane at 33 V and blocked in 5% fat-free milk. Membranes were probed with anti-human occludin (1:500), ZO-1 (1:500), claudin-1 (1:500), or claudin-4 (1:500) antibodies (Zymed) in Phosphate-buffered saline-Tween-20 (PBS-T). Proteins were visualized with a peroxidase-conjugated secondary antibody (1:10,000) by enhanced chemiluminescence (ECL).

Immunofluorescence and confocal microscopy. To determine the effects of scrambled peptide and occludin peptide on the localization of occludin, ZO-1, claudin-1, and claudin-4, indirect immunofluorescence was performed on vehicle-treated, scrambled peptide-treated and occludin peptide-treated cultures at 24 h following peptide treatment. Cells were permeabilized with methanol at -20°C for 10 min and re-hydrated 3 x 10 min with PBS. Cells were then blocked with 1X PBS containing 5% BSA (+0.5% Triton X-100 for claudin-1 staining) for 30 min at room temperature. Following 3 washes in 1X PBS, antibodies to occludin, ZO-1, claudin-1, and claudin-4 (Zymed) at a dilution of 1:1000 were added to the apical surface for 1 hr. Cells were washed with PBS and Alexa–labeled secondary antibodies (Amersham), diluted to 10 µg/ml in 10% goat serum/PBS, were added to the apical surface and incubated for 1 hr at room temperature.
Cells were post fixed in 4% paraformaldehyde and images captured with a Zeiss 510 laser-scanning microscope.

**Measurement of fence function.** To determine the effect of occludin peptide on membrane integrity, fence function was evaluated by assessing intramembrane diffusion of BODIPY-sphingomyelin. The apical domains of filter-grown WD HAE cells were labeled with BODIPY-sphingomyelin/BSA complexes that were prepared in P-buffer (10 mM Hepes, pH 7.4, 145 mM NaCl, 1 mM Na-pyruvate, 10 mM glucose, 3 mM CaCl$_2$) by slowly mixing 10 ml of 0.8 mg/ml defatted BSA (Sigma) with 200µl of BODIPY-FL-C5-sphingomyelin (Molecular Probes, Inc.) stock solution (1 mM in DMSO) under vigorous vortexing. Cells were subsequently labeled with 1:2 diluted BODIPY-sphingomyelin/BSA complexes for 10 min on ice. After the cells were washed four times with P-buffer, they were mounted on an optical chamber and images were captured in an XZ plane by confocal microscopy. Occludin peptide at 1000 µM was applied to the lumen and serial images captured at 30 sec, 1, 2, 5, 10, 20 and 30 min following occludin peptide application.

**Statistical Analysis.** Data are presented as means ± S.E.M. A one-way analysis of variance (ANOVA) and Holm-Sidak's method for all pairwise multiple comparison procedures were used to determine statistical significance of observed differences ($P<0.05$).
RESULTS

Effect of occludin peptide on Permeability. To determine whether apically delivered occludin peptide could alter permeability in WD HAE cells, the effects on transepithelial resistance were analyzed. Following apical exposure of WD HAE to 10, 30, 100, 300 or 1000 μM concentrations of occludin peptide, $R_T$ rapidly decreased in a dose-dependent manner. An 84% decrease in $R_T$ was induced by 1000 μM occludin peptide within 15 min of occludin peptide application ($P<0.001$), while a 300 μM concentration induced a 63% decrease ($P<0.001$) compared to initial $R_T$ values (Figure 2). Rapid decreases in $R_T$ were also observed with 10, 30, and 100 μM concentrations of occludin peptide. By 24 hr post occludin peptide-treatment, $R_T$ recovered to normal levels in all treatment groups, similar to the levels observed prior to occludin peptide-treatment. No significant changes in $R_T$ were observed with the apical application of 300 μM or 1000 μM of a scrambled peptide, suggesting that the rapid decrease observed in 300 μM and 1000 μM occludin peptide-treated cells was specific for the active peptide and not vehicle or lipoamino acid induced. Our data also suggest that occludin peptide induced a reversible and concentration-dependent decrease in $R_T$. Since maximum decreases in $R_T$ with full recovery were observed with the two highest concentrations of occludin peptide (300 μM and 1000 μM), these two concentrations were selected for all subsequent experiments.

To determine the effect of occludin peptide on tight junction permeability to non-polar solutes in WD HAE cells, the paracellular flux of 70- or 2000 kDa FITC-labeled dextrans following apical exposure to 300 or 1000 μM occludin peptide was measured. The basal permeability to the 70 kDa dextran was higher than that of 2000 kDa dextran by more than 15-fold (Figure 3A and 3B). Following treatment with 1000 μM occludin
peptide, permeability was similar for both dextrans, increasing by more than 4-fold to 70 kDa dextran ($P<0.001$) and more than 100-fold to 2000 kDa dextran ($P<0.001$). The smaller fold increase in paracellular permeability for the 70 kDa dextran reflects the greater basal permeability of cultures to 70 kDa dextran as compared to the 2000 kDa dextran. At 24 hr post occludin peptide application, the paracellular permeability to both 70- and 2000 kDa dextrans returned to levels of permeability measured in vehicle and scrambled peptide (1000 µM) control cultures, suggesting the increase in paracellular permeability to large dextrans is transient and reversible.

**Effect of occludin peptide on gene transfer efficiency.** To determine whether the occludin peptide could increase tight junction permeability to biologically relevant macromolecules such as gene transfer vectors, the transduction efficiencies of an adenoviral vector encoding a LacZ transgene (AdLacZ), and an adeno-associated viral vector encoding a GFP transgene (AAV2 U1a GFP) were evaluated in WD HAE cells apically exposed to 300 µM and 1000 µM occludin peptide, the two highest concentrations that rapidly reduced transepithelial resistance. The effect of 1000 µM of a scrambled peptide on AdLacZ gene transfer efficiency was also evaluated. Similar to our results with molecular tracers, pretreatment of WD HAE cells with, 1000 µM occludin peptide significantly enhanced the transduction efficiencies of both AdLacZ (MOI 300) and AAV2 U1a GFP (MOI 500) vectors compared with vehicle control cultures or 1000 µM scrambled peptide treated cells, as assessed by X-gal staining (Figure 4A) and GFP fluorescent imaging (Figure 4B) respectively. Enzymatic analysis of the level of Ad-mediated β-galactosidase expression showed significantly higher levels of this protein in cultures pre-treated apically with 1000 µM occludin peptide compared to that of vehicle-
treated or scrambled peptide-treated control cultures transduced with the AdLacZ vector alone (Figure 4C). Vehicle control cultures and scrambled-peptide treated cells had β-galactosidase activity levels of 208 ± 24 mU and 218 ± 35 mU of β-galactosidase/mg of protein respectively, whereas 300 µM occludin peptide-treated cultures had an activity of 419 ± 110 mU β-galactosidase/mg protein that exhibited a trend, but were not significantly different from vehicle or scrambled peptide treated cultures. However, 1000 µM occludin peptide-treated cultures exhibited a significantly enhanced mean activity of 7215 ± 912 mU β-galactosidase/mg protein (P<0.001). Thus, apical pre-treatment of WD HAE cells with 1000 µM occludin peptide significantly enhanced tight junction permeability, allowing for increased penetration of both Ad- and AAV-vectors to the basolateral membrane where the viral receptors are localized (Walters et al., 1999), resulting in greater viral binding and internalization.

**Effect of occludin peptide on cellular toxicity.** To determine whether the occludin peptide-induced alterations in tight junction permeability resulted in cellular toxicity, we measured lactate dehydrogenase (LDH) release into the culture media at 6 and 24 hr after apical application of occludin peptide in WD HAE cells. This assay is a relative measure of the amount of LDH release into the media compared to control cultures. Toxicity was observed only with the highest occludin peptide concentration of 1000 µM at 6 hr post treatment, with a mean occludin peptide-induced LDH release of 8%, compared with 2.25% LDH release in vehicle control cultures (P<0.001). The 300µM concentration of occludin peptide did not significantly increase LDH release as compared to vehicle control cultures (Figure 5A). A similar trend was observed in a subsequent experiment when a relative comparison in LDH levels was performed in scrambled peptide treated...
and 1000 µM occludin peptide-treated cells at 6 hr and 24 hr post peptide application (Figure 5B). No difference in the amount of LDH was observed in cells exposed to the scrambled peptide compared to vehicle treated cultures. The high baseline levels of LDH observed in the second experiment could be due to a higher concentration of cells used, or due to variations in the serum and other factors in the culture media that may have an LDH activity. By 24 hr post treatment, LDH levels in all occludin peptide-treated cultures were comparable to LDH levels in scrambled peptide-treated or vehicle control cells.

**Effect of occludin peptide on cellular permeability.** To determine whether the increase in permeability to dextrans and viral vectors was associated with increased cellular rather than paracellular permeability, we assessed solute permeability in live occludin peptide-treated WD HAE cultures with Texas Red labeled 70 kDa dextrans and XZ confocal microscopy scans. Although 70 kDa dextrans are fairly large molecules, it has previously been demonstrated that even larger molecular tracers such as 2000 kDa dextrans can enter into permeabilized HAE cells (Coyne et al., 2003). Therefore, we assessed the cellular uptake of fluorescently-labeled 70 kDa dextrans in occludin peptide-treated cultures as a measure of transcellular permeability. Our results showed that 1000 µM occludin peptide application did not induce cellular uptake of the fluorescently labeled dextran, since no fluorescence was detected within the epithelial cells for up to 30 min post occludin peptide application (Figure 6). The gradual increase in Texas Red-labeled dextrans in lateral and basal regions surrounding the epithelial cells by 30 min post occludin peptide treatment suggests an increase in paracellular flow of dextrans across the epithelium. No uptake in columnar cells was detected and infrequent uptake...
into basal cells was observed. A similar trend was observed with 300 µM occludin peptide application, although the extent of paracellular diffusion of fluorescently labeled dextrans was reduced. These data suggested that apical application of 1000 µM occludin peptide does not permeabilize the apical cell membrane, and that the observed increase in LDH release was not associated with increased cellular permeability to large molecular tracers. No difference in Texas Red dextran permeability was observed with the apical application of 1000 µM of the scrambled peptide compared to vehicle control cultures. Since it has been previously demonstrated that the sodium salt of the medium chain fatty acid, capric acid (C10) alters tight junction barrier function in epithelial cells (Anderberg et al., 1993; Coyne et al., 2003), a control experiment using C10 was performed. Cellular uptake of the 70 kDa dextran into the epithelium was observed within 1 min of apical application of C10, which was not detected in occludin peptide-treated cells.

**Specificity of occludin peptide on tight junction protein expression and localization.**

To determine whether tight junction modulation by apical exposure of WD HAE cells to occludin peptide was a result of the specific effects on occludin, alterations in tight junction-associated protein expression and distribution were assessed by Western blot analyses and immunofluorescent localization of occludin, claudin 1, claudin 4 and ZO-1 in control and occludin peptide-treated cultures. No consistent changes in the total amounts of occludin, claudin 1, or claudin 4 were detected by Western blotting in WD HAE cells immediately following or 24 hr following occludin peptide treatment (Figure 7A and 7B respectively). However, in WD HAE cells apically exposed to 1000 µM occludin peptide, there were subtle changes in the redistribution of occludin and ZO-1 immediately following occludin peptide application, with some loss of the chicken wire
occludin staining pattern in the XY plane of occludin peptide-treated, but not control cultures (Figure 7C). Alterations in the localization of claudins 1 and 4 at a similar time point were less prominent, if any. Immunofluorescent localization of occludin, ZO-1, claudin 1, and claudin 4 showed no changes in their distribution at 24 hr following apical exposure to occludin peptide. No changes in the expression or localization of these tight junction proteins was observed in cultures apically treated with 1000 µM of the scrambled peptide. These data suggest that although treatment with the occludin peptide significantly reduces $R_T$, it does not cause a significant cellular redistribution in the tight junction proteins of polarized WD HAE cells. Since ZO-1 colocalizes with occludin, and is known to bind occludin, changes in ZO-1 localization immediately following occludin peptide application likely resulted from the subtle redistribution of occludin.

Effect of occludin peptide on tight junction fence function. Although the redistribution of occludin immediately following occludin peptide application did not appear to be dramatic, we further assessed the specificity of occludin disruption by measuring the fence function in occludin peptide-treated and control cultures. The apical membrane lipids of WD HAE cells were labeled with BODIPY-sphingomyelin and changes in apical membrane lipids before and after addition of vehicle, scrambled peptide, or occludin peptide were measured. Although very subtle disruptions of the apical membrane at 30 min following occludin peptide application may have occurred, the fluorescent-labeled lipid generally remained confined to the apical domain of the plasma membrane and did not diffuse to the lateral membrane (Figure 8), suggesting that apical treatment of primary airway epithelia with a high concentration of occludin peptide does not significantly alter the fence function of tight junctions even though occludin
expression remained unaltered. A relative comparison with an altered fence function using a medium chain fatty acid, capric acid (C10) demonstrated that no significant alterations in the fence function of tight junctions occurred after occludin peptide application relative to the C10 positive control.
DISCUSSION

Occludin was the first transmembrane protein of tight junctions that was identified (Furuse et al., 1993). Several lines of evidence show that occludin plays an important role in tight junction functions. Overexpression of chicken occludin in MDCK cells was shown to increase tight junction strand number with a corresponding elevation in transepithelial resistance (McCarthy et al., 1996). A truncated form of occludin was shown to increase tight junction permeability to low molecular weight molecular tracers in MDCK cells (Balda et al., 1996). Recently, suppression of occludin by stable expression of short interfering RNA was associated with changes in the gate functions of tight junctions in MDCK cells (Yu et al., 2005).

Synthetic occludin peptides provide further evidence for its role in tight junction functions. The transepithelial resistance of Xenopus epithelial cells in vitro was shown to decrease by the administration of a synthetic occludin peptide into the culture medium (Wong and Gumbiner, 1997). Peptides corresponding to the first extracellular loop of occludin also increased the tight junction permeability to mannitol in Caco-2 cells (Tavelin et al., 2003), suggesting that occludin is a good target for tight junction modulation. Nothing is known about the effect of synthetic occludin peptides on tight junction permeability in airway epithelial cells. In this study, we investigated the effects of apical application of occludin peptide on paracellular permeability in WD HAE cells.

Our study showed that occludin peptide rapidly decreased $R_T$ in WD HAE cells, with a significant reduction within minutes of apical exposure to 300 or 1000 µM occludin peptide. The occludin peptide-induced decrease in $R_T$ was reversible and returned to normal levels within 24 hr post confirming the reversibility of this strategy.
The significant decrease in $R_T$ resulted in an alteration in tight junction permeability as evidenced by a corresponding increase in paracellular permeability to molecular tracers. Significant increases in the diffusion of both low and high molecular weight dextrans were observed in primary airway epithelia previously treated with 1000 µM occludin peptide. To further test the effects of the occludin peptide on tight junction permeability to macromolecules, we measured the transduction efficiencies of adenoviral and adeno-associated viral vectors in WD HAE cells pre-exposed to apical occludin peptide. Significant increases in gene transfer mediated by both adenoviral and AAV vectors were detected in occludin peptide-treated WD HAE cells. This finding suggested that occludin peptide alters paracellular permeability and increases translocation of vectors to the basolateral membrane, resulting in enhanced binding of viral vectors to viral receptors.

Although it has been previously demonstrated that EGTA and the medium chain fatty acids C10 and C12 can enhance dextran diffusion and gene transfer efficiency (Johnson et al., 2003; Gregory et al., 2003), these agents have been shown to have varying toxicity profiles. C12 is toxic to airway epithelial cells, as evidenced by the presence of high levels of LDH in the culture media even at 72 hr post C12 treatment. Our data with apical delivery of occludin peptide showed minimal, transient toxicity with the highest concentration of 1000 µM at 6 hr post-treatment, which was resolved by 24 hr. The transient release of LDH raised the question whether the increase in permeability to dextrans and viral vectors following occludin peptide application was due to transcellular permeability rather than an increase in tight junction permeability. To address this question, we analyzed Texas Red dextran permeability in live WD HAE cells.
following occludin peptide-treatment (Figure 6). Penetration of a fluorescently labeled 70-kDa dextran in live WD HAE cultures was visualized by scanning images in the XZ plane by confocal microscopy. The apical application of 1000 µM occludin peptide did not result in cellular uptake throughout the epithelium evidenced by the absence of fluorescence within the epithelial cells even at 30 min post occludin peptide-treatment. XZ images captured at various time points following occludin peptide treatment showed the passage of Texas Red labeled dextrans through the paracellular, rather than the transcellular pathway. Similar results were observed with 300 µM occludin peptide treatment.

When the apical membranes of the WD HAE cells were labeled with BODIPY-sphingomyelin followed by occludin peptide treatment, a slight dissipation of label intensity at the apical membrane was detected in occludin peptide-treated, but not control cultures. This loss of intensity may have accounted for the increased LDH release since the lipophilic amino acid may transiently interact with the apical membrane to release the peptide from the prodrug complex (lipoamino acid plus peptide). This interaction might increase the release of small molecules such as LDH, but not increase permeability to large dextrans. Although we could not exclude the possibility that some BODIPY-sphingomyelin was detected in the lateral membranes of occludin peptide-treated cells due to exchange of some apical membrane BODIPY-sphingomyelin into the P-buffer, followed by reinsertion into the lateral membrane, we did not readily detect lateral diffusion of BODIPY-sphingomyelin in occludin peptide-treated cells. As a control for diffusion of BODIPY-sphingomyelin from the apical to the lateral surface, a relative comparison to altered fence function induced by sodium caprate (C10) was performed indicating lateral diffusion of labeled membrane lipid in C10-treated cultures, whereas no
significant diffusion of label to the lateral membrane space was detected on apical application of occludin peptide.

Thus, exposure of WD HAE cells to 1000 µM occludin peptide resulted in a mild, transient increase in LDH release that was not associated with significant alterations in the structural components of the epithelia (Fig. 6, 8). The high levels of LDH in the culture media of vehicle control cells at 24 hr possibly resulted from the cumulative loss of cells in these terminally differentiated cultures that typically senesce at ~8 weeks after plating. Ciliated cells are also highly metabolic and deplete energy stores while secreting lactate which may in turn affect cell viability. However, the key point is that the LDH levels were similar in both treatment and control (scrambled peptide and vehicle) groups at 24 hr, suggesting that the toxicity induced by high concentrations of occludin peptide is transient, and quickly resolves.

To further determine whether occludin peptide application affected the expression and distribution of tight junction proteins, Western blot analysis and immunolocalization of tight junction-associated proteins was performed on control and occludin peptide-treated cultures. Immunolocalization analysis immediately following occludin peptide application showed a subtle redistribution of occludin and ZO-1, with no significant changes in claudin 1 and claudin 4 localization. Changes in the distribution of occludin and ZO-1 may be due to specific targeting of occludin by the occludin peptide, resulting in disruption of the occludin-ZO-1 complex found at tight junctions. Although IFN-gamma increases paracellular permeability in intestinal epithelial cells by inducing endocytosis of occludin, JAM-A, and claudin-1, assessment of whether this mechanism
occurred during enhanced paracellular permeability following occludin peptide application was beyond the scope of this study.

At 24 hr following apical application of occludin peptide there was no evidence of redistribution of any of these tight junction proteins. Furthermore, no significant alterations in the expression of occludin, claudin-1 and claudin-4 were observed in Western blot analyses of WD HAE cells immediately after or 24 hr following apical exposure to occludin peptide. The lack of significant changes in these tight junction-protein components suggests that the occludin peptide-induced alterations in tight junction permeability were primarily due to specific disruption of occludin.

$C_{14}-OP_{90-103}$ used in this study is a racemic mixture of two diastereomers, $d$-$C_{14}$-$OP_{90-103}$ and $l$-$C_{14}$-$OP_{90-103}$, which vary in their stability (Toth et al., 1999; Tavelin et al. 2003). The $l$-isomer containing $L$-2-amino dodecanoic acid ($L$-$C_{14}$-$OP$) not only released $OP_{90-103}$ at a 15-fold faster rate than the $d$-isomer that contained $D$-2-amino dodecanoic acid ($D$-$C_{14}$-$OP$), but also decreased $R_T$ by ~40-fold (Tavelin et al., 2003). Since only intact $OP_{90-103}$ was released by either isomer, it indicated that the lipoamino acid moiety prevented the released $OP_{90-103}$ from degradation. In the present study, a high concentration of occludin peptide was required to increase paracellular permeability to dextrans and gene transfer vectors. However, this high concentration resulted in modest cellular toxicity in occludin peptide-treated WD HAE cells. Use of the more active $l$-isomer of $C_{14}$-$OP_{90-103}$ rather than a mixture of the both the $d$-$C_{14}$-$OP_{90-103}$ and $l$-$C_{14}$-$OP_{90-103}$ isomers may be a safer alternative to effectively modulate tight junction permeability with minimal toxicity.
Based on our results, apical occludin peptides may represent a better class of tight
junction modulators in airway epithelial cells that specifically target the extracellular
domains of tight junction proteins resulting in enhanced tight junction permeability with
minimal toxicity. Although further studies to elucidate the safety profile of occludin
peptide in lung epithelia are needed, specific modulation of tight junctions by occludin
peptide could prove to be a valuable alternative strategy for the efficient delivery of
pharmacological agents and viral vectors for the treatment of lung diseases.
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REFERENCES


Footnotes

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**FIGURE LEGENDS**

**Fig. 1.** The occludin peptide OP$_{90-103}$ consisting of the amino acids DRGYGTSLLGGSVG was conjugated to a racemic 2-amino-tetradecanoic acid (C$_{14}$) moiety that was added to the N terminus of occludin peptide, resulting in C$_{14}$-OP.

**Fig. 2.** Effects of occludin peptide on transepithelial resistance ($R_T$). $R_T$ of WD HAE cells before and after treatment with 300 and 1000 $\mu$M scrambled peptide, 10, 30, 100, 300 and 1000 $\mu$M concentrations of occludin peptide, and vehicle (no peptide) control cultures, measured at 10 min intervals up to 30 min, and at 60 min, 90 min, 6hr, 24 hr and 48 hr. $R_T$ recovery was monitored up to 48 hr following removal of occludin peptide at the 30 min time point (n=3 cultures for 300 and 1000 $\mu$M scrambled peptide treated cultures, n=6 for all other treatment cultures).

**Fig. 3.** Effects of apically delivered occludin peptide on tight junction permeability to (A) a 70 kDa FITC-labeled dextran and (B) a 2000-kDa FITC-labeled dextran at 60 min and at 24 hr following occludin peptide application. Permeability is expressed as paracellular permeability coefficient ($P_{app}$). Values are given as mean values ± S.E.M. (n = 3 cultures for the scrambled peptide-treated group; n=6 cultures for all other treatment groups). *Significantly different from vehicle and scrambled peptide-treated control cultures.

**Fig. 4.** Effect of apically delivered occludin peptide on gene transfer efficiency at 48 hr post infection. (A) AdlacZ transduction in vehicle (no peptide) WD HAE control cultures or following treatment with 1000 $\mu$M scrambled peptide, 300 $\mu$M occludin peptide or 1000 $\mu$M occludin peptide. (B) AAV2 U1a GFP transduction in vehicle WD HAE control cultures or following treatment with 300 $\mu$M or 1000 $\mu$M occludin peptide.
(C) LacZ transgene expression assessed by a β-Galactosidase enzymatic assay in vehicle, scrambled peptide- and occludin peptide-treated WD HAE cells infected with the AdLacZ vector. Values are given as mean values ± S.EM. (n = 3 cultures per scrambled peptide treated group, n=6 for all other treatment groups). *Significantly different from vehicle and scrambled peptide-treated control cultures.

**Fig. 5.** Release of LDH into culture media at 6 hr and 24 hr in vehicle treated WD HAE cultures or following treatment with 300 µM occludin peptide or 1000 µM occludin peptide (A), and in vehicle-treated, 1000 µM scrambled peptide-treated and 1000 µM occludin peptide-treated cells (B). LDH release is represented as mean percent occludin peptide-induced LDH release. * denotes a significant difference compared to control cultures. Values given are mean values ± S.E.M. (n = 9 cultures for vehicle control cultures and 300 µM occludin peptide-treated groups; n = 3 for 1000 µM SP-treated cultures; n=15 cultures for 1000 µM occludin peptide-treated group).

**Fig. 6.** Imaging of 70 kDa Texas Red dextran permeability in live WD HAE cultures at 0, 1 and 30 min following apical application of 300 or 1000 µM occludin peptide, and at 0, 1 and 2 min following apical application of 30 mM C10. Phosphate buffered saline containing 2 mg/ml Texas Red labeled 70 kDa dextran was apically applied to WD HAE cells followed by the addition of occludin peptide or C10. Scans (magnification 400X) were taken along the XZ axis at the denoted time points by confocal microscopy. A magnified image of the paracellular flow of Texas Red dextran at 30 min following 1000 µM occludin peptide is shown below.

**Fig. 7.** Western blot detection of occludin, claudin 1, and claudin 4 expression (A) immediately following apical application of scrambled or occludin peptides and (B) at 24
hr following exposure to scrambled and occludin peptides in WD HAE cultures. Each lane is representative of a treatment group (n=3 for scrambled peptide and n=6 for all other treatment groups). (C) Immunofluorescent localization of occludin, ZO-1, claudin 1, and claudin 4 immediately following apical application of the scrambled peptide or occludin peptide and at 24 hr post occludin peptide-application in WD HAE cells. Vehicle treated cultures were used as the control group. Images (400X magnification) are representative of 3 cultures per treatment group for the scrambled peptide and 6 cultures for the occludin peptide. All images were captured under the same confocal conditions. The bar scale represents 20 µm.

Fig. 8. Analysis of intramembrane diffusion of BODIPY-sphingomyelin by confocal microscopy. The apical domains of WD HAE cells were labeled with BODIPY-sphingomyelin/BSA complexes and diffusion of lipids from the apical to the basolateral cell domain were analyzed at 0, 1, 15 and 30 min in vehicle treated cultures or following apical application of 1000 µM occludin peptide. Shown are XZ images at 400X magnification (n=3 per treatment group). A C10 treated culture was used as a positive control.
Figure 2
Figure 3
Figure 4
Figure 5
Figure 6