NOVEL EFFECTS OF AZITHROMYCIN ON TIGHT JUNCTION PROTEINS IN HUMAN AIRWAY EPITHELIA

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ABSTRACT

The macrolide antibiotic azithromycin improves lung function and prognosis among patients with cystic fibrosis or diffuse panbronchiolitis, independent of bacterial eradication. Anti-inflammatory effects have been implicated, but data from *in vivo* studies are scarce and the link between abnormal electrolyte content in airway surface liquid and bronchial infections remains uncertain.

In the present study we treated human airway epithelia on filter supports with azithromycin and monitored transepithelial electrical resistance. We found that azithromycin increased transepithelial electrical resistance of airway epithelia in a dose dependent manner. Immunocytochemistry and western blot analysis showed that addition of azithromycin changed protein location in cell cultures and induced processing of tight juction proteins; claudin-1 and –4, occludin and junctional adhesion molecule-A. These effects were reversible and no effect was seen when cells were treated with penicillin or erythromycin. The data indicate that azithromycin increases transepithelial electrical resistance of human airway epithelia by changing the processing of tight junction proteins. The results are novel and may help explain the beneficial effects of azithromycin in patients with cystic fibrosis, diffuse panbronchiolitis and community acquired pneumonia.

INTRODUCTION

Respiratory infections remain an important cause of morbidity and mortality despite the development of novel antimicrobial agents. New infectious agents emerge and multi-resistant bacteria are a growing problem leading to increased interest in host defense research that could provide tools in the fight against pulmonary infections. Epidemiological studies have generated interesting results regarding lung defense mechanisms. An example is the effect of macrolides, a class of commonly prescribed antibiotics, on patients with respiratory infections. Treatment with macrolide antibiotics improved 5- and 10 year survival among patients with diffuse panbronchiolitis (10, 20). This observation set the stage for large studies evaluating the effect of macrolide antibiotics on patients with cystic fibrosis (CF). Three recent randomized, placebo-controlled trials indicate that azithromycin significantly improves lung function by increasing forced expiratory volume in one second (5, 18, 28). Interestingly, improvement in lung function did not correlate with reduction of *Pseudomonas aeruginosa* or *Staphylococcus aureus* in sputum, suggesting that the favorable effect of azithromycin did not require bacterial eradication (5, 18, 28). Other studies found that the combination of a macrolide and a cephalosporin antibiotic improved the prognosis of patients with pneumococcal pneumonia compared to single-antibiotic treatment (12, 26). Cephalosporins are active against *Streptococcus pneumoniae* but the beneficial effect of additional macrolide therapy on patients with this common type of pneumonia is largely unexplained. Speculations regarding the mechanism by which azithromycin improves clinical outcome in diffuse panbronchiolitis, CF and pneumonia include an antiinflammatory effect, effect on sputum rheology, biofilm formation, bacterial adherence and flagellin expression (16, 17, 24) Several studies have demonstrated the importance of the bronchial epithelium

in lung defense (13, 23). In addition to being a mechanical barrier it regulates electrolyte content of the airway surface liquid (ASL) (25). In cystic fibrosis, loss of cystic fibrosis transmembrane conductance regulator (CFTR) chloride channel activity produces abnormal ASL electrolyte and water content. Data also indicate that this may decrease lung defense against infections (23). If the electrolyte and water content of the ASL does affect lung defense, regulation of ion transport through paracellular pathways could be important in preventing lung infections. Tight junctions (TJs) located in the apicolateral membrane of epithelia, form a barrier between adjacent cells and regulate the movement of ions and solutes across the paracellular space. TJs vary among different epithelia in barrier properties, meeting different functional requirements for each tissue type. The TJ complex consists of three types of transmembrane proteins; claudins, occludin and junctional adhesion molecules (JAMs) as well as zonula occludens (ZO) proteins that serve as adaptors to the actin cytoskeleton at the cytoplasmic face of TJs (14). Claudins and occludin are tetraspan transmembrane proteins with two extracellular loops and cytoplasmic C- and N-termini. There are at least 24 members of the claudin family predicted to range in size from 20 to 27 kDa and they show distinct tissue expression patterns. Occludin is considerably larger at ~65 kDa and is widely expressed at TJs. JAM-A is a single pass transmembrane protein of ~40 kDa and a member of the immunoglobulin superfamily.

The importance of claudins as regulators of paracellular ion transport is evident in several human diseases. Simon et al. showed that mutations in the gene encoding claudin-16 is the cause of recessive renal hypomagnesemia. Their data further suggested that claudins could form a selective paracellular ion channel (22). Other studies indicate that claudins-3 and -4 are receptors for *Clostridium perfringens*

enterotoxin (9), a common cause of food poisoning. Mutations in claudin-14 are associated with a recessive form of deafness where the ionic environment in the cochlear duct is altered (27). To our knowledge no data are available regarding a role for occludin or JAM-A in electrolyte transport.

In this study we show that azithromycin increases transepithelial electrical resistance (TER) in human airway epithelia *in vitro* and affects both localization and processing of the tight junction proteins claudin-1, -4, occludin and JAM-A. These effects of azithromycin on TJ proteins were specific and reversible but no effects were found on the adherens junction protein E-cadherin or after treatment with other antibiotics such as penicillin. The results show novel biological effects of a commonly used antibiotic on key proteins that maintain respiratory epithelial integrity and could be the initial step explaining the clinical benefit from azithromycin treatment in CF, diffuse panbronchiolitis and community acquired pneumonia.

MATERIALS AND METHODS

Cell culture

Primary bronchial epithelial cells (a gift from prof. Michael J. Welsh, University of Iowa, Iowa City, IA) were cultured on plastic flasks coated with Vitrogen 100 (Cohesion, Palo Alto, CA) in serum- and antibiotic-free bronchial epithelial growth medium with supplements (CC3170, Cambrex, East Rutherford, NJ). We established an immortalized cell line, VA-10. Transduction of normal human bronchial epithelial cells was performed with sterile filtered supernatant from the PA317 LXSN packaging cell lines, containing retroviral construct with human papilloma virus 16 E6 and E7 (CRL-2203, American Type Culture Collection, Rockville, MD), and the neomycin resistance gene. Transduction was done in the

presence of 8 µg/ml polybrene (Sigma-Aldrich). Transfected cells were selected by cultivation in the presence of 500 µg/ml neomycin (Life Technologies, Gaithersburg, MD). For immunocytochemistry, cells were grown on Chamber Slides (Nalge Nunc, Naperville, IL). For TER experiments, cells were grown on Transwell permeable support filters (3460, Corning Costar Corporation, Acton MA) and cultured for the first day in 50:50 DMEM-Ham´s F-12 medium (Gibco, Burlington, Canada) in 5% fetal bovine serum (Gibco). On the day after seeding, the cells were cultured and maintained in 50:50 DMEM-Ham´s F-12 medium supplemented with 2% Ultroser G (Biosepra, Cergy-Saint-Christophe, France).

Antibiotics

Azithromycin (Zitromax; Pfizer ApS, Ballerup, Denmark), erythromycin lactobionate (Abboticin; Abbot, Solna, Sweden) and penicillin G (Penicillin Leo; Leo, Ballerup, Denmark) were dissolved as instructed by the manufacturer and then further diluted to the desired concentrations.

Measurement of transepithelial electrical resistance (TER)

A Millicell-ERS voltohmmeter (Millipore, Billerica, MA) was used to measure the TER value of confluent filters. All measurements were done in triplicate and TER values were normalized for the area of the filter and were obtained after background subtraction.

Growth curve

Analysis of cell growth was performed using a standard protocol. Cells were plated onto 24 well plates and cultured at 37°C in a humidified 5% CO_2 atmosphere with or

without 40 µg/ml azithromycin. After 24 h, three wells of both cultures were trypsinized and counted by using a hemocytometer. This was repeated daily for seven days and the results plotted as a growth curve.

Immunocytochemistry

Immunofluorescent stainings were performed on methanol fixed cells. Primary antibodies were as follows: Rabbit anti-JAM-A and -claudin-1, mouse anti-claudin-4, -occludin and -E-cadherin antibodies were purchased from Zymed Laboratories (San Francisco, CA). We used iso-type specific Alexa Fluor® secondary antibody conjugates from Molecular Probes (Eugene, OR). Images were captured by Zeiss LSM 5 Pascal Confocal Microscope (Carl Zeiss AG, Munich, Germany).

Western blot

Equal amounts of proteins, as determined by Bradford method (2), were loaded and run on a NuPAGE 10% Bis-Tris gel (Invitrogen, Carlsbad, CA) and transferred to a polyvinylidene difluoride membrane (Invitrogen). The blots were blocked in 5% non-fat milk and subsequently incubated with the primary antibody overnight followed by an incubation with secondary antibodies, horseradish peroxidase-conjugated anti-mouse or rabbit for 1 h (Amersham Biosciences UK Ltd., Little Chalfont, England). Protein bands were visualized using enhanced chemiluminesecence system and Hyperfilm (Amersham Biosciences).

Statistical analysis

Statistical analaysis was performed using Student´s T-test. Data are mean ± SEM. P values <0.05 were considered statistically significant.

RESULTS AND DISCUSSION

Azithromycin increases transepithelial electrical resistance in human airway epithelia *in vitro*.

We measured TER across airway epithelia after treatment with azithromycin. We used 0.4, 4 and 40 µg/ml of azithromycin based on clinical studies showing that in patients receiving 250 mg azithromycin daily for four weeks, the median sputum concentration of azithromycin was 9,5 µg/ml (range 0.6 – 79.3 µg/ml) (1). We found that addition of 40 µg/ml azithromycin to the basolateral side of the epithelium increased TER from 1234±29 (control) to 2920±195 Ω cm^2 ± SEM ($P<0.05$, n=24) (Fig. 1A). Addition of azithromycin to the apical side had no effect on TER (data not shown). Fig. 1B shows that a single dose of 40 µg/ml azithromycin daily over 4 days increased TER by approximately 80%. Erythromycin (30 µg/ml) or penicillin (20 µg/ml) had no effect on TER (data not shown). To explore the possibility that azithromycin produced multiple layers of epithelial cells, we generated a growth curve and found that azithromycin treatment resulted in fewer cells (Fig. 1C) suggesting that proliferation of epithelial cells does not explain the observed increase in TER. Azithromycin did not affect viability and no effect on apoptosis was observed as measured by immunostaining and western blot for cleaved caspase-3 (data not shown).

Azithromycin changes the processing of claudin-1 and -4.

Tight junction proteins are required for epithelial integrity, a key component of structural and functional lung defense (15). We used specific antibodies to determine the cellular location of claudin-1 and -4. Epithelia were cultured on glass

slides and treated with azithromycin continuously from seeding, or after reaching confluence. Under both conditions, immunocytochemistry suggested that azithromycin shifted claudin-1 and -4 to an intracellular location (Fig. 2A). To further characterize this effect we used western blot analysis. Lysates from cells produced a band consistent with the molecular weight of claudins (~23 kDa). Interestingly, a rapidly migrating band (~10 kDa) in addition to the expected 23 kDa band (Fig. 2B) was produced after treatment with azithromycin (20 and 40 µg/ml). The smaller sized band was detected both with claudins-1 and -4. These data indicate that azithromycin affects the processing of claudin-1 and -4. Azithromycin produced the same processing pattern for claudin-1 in two other cell lines, the alveolar epithelial A549 and the breast luminal epithelial D382 (data not shown) suggesting that this effect is general in epithelial cells. The processing of TJ proteins could affect lung defense mechanisms such as the mechanical barrier function or the regulation of airway surface liquid electrolytes. Interestingly, earlier studies suggest that azithromycin improves outcome in patients with CF, diffuse panbronchiolitis and pneumonia, independent of antibacterial effects. The data presented here might help explain some of the beneficial clinical effects of azithromycin. Unlike azithomycin, penicillin or erythromycin did not affect the processing of claudin-1 (Fig. 2C).

One potential mechanism by which azithomycin might alter the processing of tight junction proteins is activation of proteolytic enzymes. The size of the rapidly migrating band in the claudin experiments could be consistent with a cleavage site in the cytoplasmic loop. This could affect the structure or location of the extracellular loops of claudins that have been shown to determine charge selectivity and TER (3, 4). However, several other possibilities exist and the origin of the rapidly migrating band requires further analysis by immunoprecipitation and amino acid sequencing.

Azithromycin changes the processing of occludin and JAM-A.

To test the possibility that azithromycin affected the processing of occludin, JAM-A or E-cadherin, we used immunocytochemistry and western blot analysis. Figure 3A indicates that azithromycin induces intracellular location of occludin and JAM-A but does not affect the location of E-cadherin. Analysis of occludin protein expression revealed a ~65 kDa band consistent with the molecular weight of full length occludin (Fig. 3B). Interestingly, a shift towards a smaller sized band ~40 kDa was observed in lysates from cells treated with azithromycin. Azithromycin also affected the expression of JAM-A, producing two rapidly migrating bands in addition to the expected 36-41 kDa band. In contrast, the western blot of E-cadherin was unaffected by azithromycin (Fig. 3B).

The intracellular accumulation of occludin, claudins and JAM-A after treatment with azithromycin is a puzzling phenomenon. Protein retention in endoplasmic reticulum or in Golgi apparatus, allowing cleavage, is a potential explanation for the effect of azithromycin on the processing of TJ proteins (Fig. 2A and 3A). Recent studies by Howe et al. demonstrate that cells treated with TGFβ resulted in perinuclear accumulation of the CFTR chloride channel in epithelial cells. This was shown to be dependent on reorganization of the actin cytoskeleton. Exposure to TGFβ caused reorganization of F-actin into elongated stress fibers, in marked contrast to the more diffuse F-actin in control epithelial cells (8). Since the TJ complex is linked to the actin cytoskeleton through ZO proteins, future studies should address this issue.

The expression of nonjunctional cell adhesion molecules may be affected by azithromycin. Semaan et al. found no significant effect of azithromycin on plasma

levels of sICAM in patients with coronary artery disease (21). In contrast, Hillis et al. showed that a 5-day azithromycin course in patients recovering from an acute coronary syndrome reduced serum levels of sICAM-1 (7)**.** By studying the effect of azithromycin on nonjunctional cell adhesion molecules we could have made our observations more specific. However, our data showing that azithromycin does not affect the processing of E-cadherin suggest that its effect on claudin-1, -4, occludin and JAM-A is specific. In addition, our model focuses on transepithelial electrolyte transport and tight junction proteins.

Interestingly, erythromycin neither affected TER nor the processing of TJ proteins. This suggests that unlike the antiinflammatory effects of macrolides, the effects on TER and the processing of TJ proteins found in our study are specific to azithromycin. Azithromycin is derived from erythromycin, the chemical difference is a methyl-substituted nitrogen atom incorporated into the lactone ring. Wheather this is required in the macrolide chemical structure to affect the processing of TJ proteins should be further investigated. The successful management of DPB with erythromycin has been explained by its antibacterial and antiinflammatory effects. The etiology of DPB remains unknown. Unlike CF, DPB has not been shown to be caused by defects in transepithelial electrolyte transport. Therefore, the clinical effect of azithromycin in CF patients could be caused by its common macrolide effects in addition to its specific effects on transepithelial electrolyte transport.

The effect of azithromycin on the processing of claudin-1 and occludin is reversible.

To test if the effect of azithromycin on the processing of claudin-1 and occludin was reversible we applied azithromycin to epithelia daily for four days and

maintained the culture without azithromycin. Protein was extracted before treatment and then daily. The data show that the effect of azithromycin on claudin-1 and occludin is evident 24 h after first exposure to azithromycin. The effect is reversed at day 5, 24 h after removal of azithromycin (Fig. 4).

Conclusion

The study shows that azithromycin increases TER and affects the processing of tight junction proteins in human airway epithelia *in vitro*. The data do not define an association between altered protein processing and TER modification. However, such association is suggested by various earlier studies; the extracellular loops of claudins contain charged amino acids (19)**,** the expression of different claudins increases or decreases TER (6) and, claudins create charge-selective channels in certain epithelial paracellular pathways (4).

The effects of azithromycin reported here are novel and may have implications for lung defense. Lee et al. (11) found that confluent low-TER airway epithelia bound 25 times more *P. aeruginosa* than confluent high-TER airway epithelia and the bacterium bound frequently at cell borders, indicating that tight junctions might be involved. Claudins or other tight junction proteins are potential therapeutic targets in CF and other diseases of abnormal transepithelial ion transport. Future studies might attempt to better define the effect of azithromycin and other antibiotics on the function of tight junction proteins. Such work could be important in light of recent international pneumonia epidemics and increasing bacterial resistance to multiple antibiotics.

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FIGURE LEGENDS

Figure 1.

A. Effect of azithromycin on TER of human airway epithelia *in vitro*. Human airway epithelial cells were cultured on Transwell filters. After reaching confluence azithromycin (0.4, 4.0 and 40 µg/ml) was added to the basolateral side of epithelia every 48 h for 8 days. TER was measured using a Millicell-Electrical Resistance System. Data are mean ± SEM, n=3. Azithromycin increased TER in a dose-dependent manner.

B. Azithromycin increases TER in human airway epithelia.

Measurements were made at day 0 (open bars), before any treatment, and at day 4 (solid bars), after four doses of 40 µg/ml azithromycin. Data are mean ± SEM, n=6. Azithromycin 40 µg/ml daily increased TER significantly ($P<0.0001$).

C. Growth curve.

Human airway epithelial cells were cultured on 24-well plates and treated continuously with 40 µg/ml azithromycin. Data are mean ± SEM, n=3. Azithromycin 40 µg/ml decreases cell proliferation.

Figure 2.

A. Immunocytochemical analysis of the effect of azithromycin on the expression of claudin-1, and –4. Human airway epithelial cells were cultured on chamber slides. *Green* indicates expression of claudin-1 or -4. Left row; control. Right row; after treatment with 40 µg/ml azithromycin.

B. Western blot analysis of the effect of azithromycin on the expression of claudin-1, and –4. Equal amounts of protein from cells treated with different

concentrations of azithromycin were subjected to western blot analysis. Blotting for claudin-1 and –4 revealed a rapidly migrating band in lysates from cells treated with 40 µg/ml azihromycin.

C. Effect of penicillin and erythromycin on the expression of claudin-1. Equal amounts of protein from human airway epithelial cells treated with penicillin or erythromycin were subjected to western blot analysis. Unlike azithromycin, a rapidly migrating band was not observed.

Figure 3.

Effect of azithromycin on the expression of occludin, JAM-A and E-cadherin.

A. Immunocytochemistry. Human airway epithelial cells were cultured on chamber slides. *Green* indicates expression of junctional molecules. Left row; control. Right row; after treatment with 40 µg/ml azithromycin.

B. Equal amounts of protein from cells treated with different concentrations of azithromycin were subjected to western blot analysis. Blotting for occludin and JAM-A revealed a rapidly migrating band in lysates from cells treated with 40 µg/ml azithromycin.

Figure 4.

Reversible effect of azithromycin on claudin-1 and occludin.

Confluent cells were treated with 40 µg/ml azithromycin daily for 4 days. After day 4 cells were cultured with medium alone. Equal amounts of protein were subjected to western blot analysis. Protein was extracted before addition of azithromycin and then daily for seven days and again on day 9.