Cell Junction Remodeling in Gingival Tissue Exposed to a Microbial Toxin

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Abstract

The gingival epithelium plays a key role in protecting the supporting structures of the teeth from bacteria and their products. In *ex vivo* experiments, we recently showed that the cytolethal distending toxin (Cdt) from the periodontal pathogen *Aggregatibacter actinomycetemcomitans* causes extensive damage to gingival tissue. Morphological changes included detachment of the keratinized outer layer, distention of spinous and basal cells in the oral epithelium, disruption of rete pegs, and apparent dissolution of cell junctions. Adherens junctions (zonula adherens) are essential for maintaining barrier function and integrity of gingival epithelium. Therefore, immunohistochemical and RT-PCR analyses of human gingival explants (HGX) and human gingival epithelial cells (HGEC) were utilized for a closer examination of the effects of the Cdt on E-cadherin, the key membrane component of adherens junctions. Although there was some variability among tissue donors, exposure of gingival tissue or isolated epithelial cells to the toxin generally resulted in a pronounced increase in the expression and cytosolic distribution of E-cadherin, accompanied by an increase in levels of the intracellular scaffolding proteins β-catenin and β-actin. These results indicate that the Cdt induced substantial remodeling of adherens junctions, with a potential impact on the barrier function of gingival epithelium.

Abbreviations: cytolethal distending toxin (Cdt), 4',6-diamidino-2-phenylindole (DAPI), human gingival epithelial cells (HGEC), human gingival explants (HGX), human gingival fibroblasts (HGF), transepithelial resistance (TER).

Keywords

adherens junctions, Aggregatibacter actinomycetemcomitans, cytolethal distending toxin, E-cadherin, gingival epithelial cells, gingival explants

Disciplines

Dentistry

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Cell Junction Remodeling in Gingival Tissue Exposed to a Microbial Toxin

ABSTRACT

The gingival epithelium plays a key role in protecting the supporting structures of the teeth from bacteria and their products. In *ex vivo* experiments, we recently showed that the cytolethal distending toxin (Cdt) from the periodontal pathogen *Aggregatibacter actinomycetemcomitans* causes extensive damage to gingival tissue. Morphological changes included detachment of the keratinized outer layer, distention of spinous and basal cells in the oral epithelium, disruption of rete pegs, and apparent dissolution of cell junctions. Adherens junctions (zonula adherens) are essential for maintaining barrier function and integrity of gingival epithelium. Therefore, immunohistochemical and RT-PCR analyses of human gingival explants (HGX) and human gingival epithelial cells (HGEC) were utilized for a closer examination of the effects of the Cdt on E-cadherin, the key membrane component of adherens junctions. Although there was some variability among tissue donors, exposure of gingival tissue or isolated epithelial cells to the toxin generally resulted in a pronounced increase in the expression and cytosolic distribution of E-cadherin, accompanied by an increase in levels of the intracellular scaffolding proteins β-catenin and β-actin. These results indicate that the Cdt induced substantial remodeling of adherens junctions, with a potential impact on the barrier function of gingival epithelium.

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INTRODUCTION

Plaque-induced periodontal disease (PIPD) is initiated by a persistent mixed-bacterial infection and sustained by interactions between the microbial antagonists and host immune system (Armitage, 2004; Darveau, 2010). The gingival epithelium is an early line of defense against numerous biofilm-forming micro-organisms, creating a barrier that minimizes bacterial invasion of the underlying tissue (Gibbons, 1989). Bacterial products, along with factors released from recruited inflammatory cells, induce degradation of connective tissue and bone, altering the structural support of the teeth. Therefore, breakdown of the epithelial barrier early in the development of PIPD has significant consequences for periodontal health.

Contact between epithelial cells is stabilized by multi-protein cell junction complexes composed of transmembrane, cytosolic, and cytoskeletal proteins. These are symmetrical structures formed between cells that are critical for maintaining the physical and functional integrity of tissues. Adherens junctions (zonula adherens) are a ubiquitous form of cell junction that interacts with a core structure of intracellular scaffolding and signaling molecules (Miyaguchi, 2000; Meng and Takeichi, 2009; Kowalczyk and Nanes, 2012). Desmosomes (macula adherens) are composed of closely *cis*-packed transmembrane glycoproteins (desmogleins) that are abundant in epidermis and other stratified squamous epithelia (Franke, 2009). Tight junctions (zonula occludens) are composed of transmembrane proteins arranged head-to-head and include the claudins, occludin, and cytoplasmic adaptor proteins such as ZO-1 (Shen, 2012). Gap junctions are formed by paracrystalline connexin channels (Hirokawa and Heuser, 1981; Franke, 2009).

Aggregatibacter actinomycetemcomitans is a key periodontal pathogen thought to play a significant role in the pathogenesis of localized aggressive periodontitis (LAP; Henderson *et al.*, 2002). A secreted heterotrimeric cytolethal distending toxin (Cdt) is among its putative virulence factors. The results of *in vitro* (Lepine *et al.*, 1998; Kang *et al.*, 2005) and *ex vivo* (Damek-Poprawa *et al.*, 2011) studies have provided evidence that the toxin selectively affects the integrity and physiology of cells from human gingival tissue. Disorganization of cell-cell contact and consequent breakdown of tissue architecture are often the result of pathophysiological conditions (Franke, 2009). Therefore, our hypothesis is that Cdt-induced changes within the gingival epithelium are secondary to effects on cell-cell junctions. The goal of this study was to evaluate whether the Cdt alters the organization of adherens junction components *in situ* in a gingival explant model and in monolayers of isolated human gingival epithelial cells (HGEC).

MATERIALS & METHODS

Human Gingival Explants and Epithelial Cells

Gingival tissue (n = 19) was obtained from periodontally healthy and diseased (n = 2) adults, as described previously (Damek-Poprawa *et al.*, 2011), during routine procedures performed at the University of Pennsylvania School of Dental Medicine in accordance with an Institutional Review Board-approved protocol. All patients provided informed consent. Excised tissue was immediately processed as described (Damek-Poprawa *et al*., 2011). HGEC were isolated from periodontally healthy tissue and cultured as described.

Cdt Purification and Assembly

Recombinant Cdt subunits were isolated by affinity chromatography as described previously (Cao *et al*., 2005). Heterotrimers were reconstituted, in a refolding buffer, with equimolar amounts of wild-type sub-units (Mao and DiRienzo, 2002) or CdtB^{H160A} substituted for CdtB (DiRienzo *et al*., 2009). The TD$_{50}$ for HGEC, based on cell-cycle arrest, is 1.1 µg/mL.

Transepithelial Resistance (TER) Measurements

HGEC were seeded at a density of 5 x 10^4, in serum-free kera-tinocyte medium, on 12 mm 0.4-µm-pore-size Transwell® inserts (Corning, Lowell, MA, USA) and incubated for 3 days to allow the cells to reach confluence. TER measurements were taken by means of an EVOM^2 Epithelial Voltohmmeter (World Precision Instruments, Inc., Sarasota, FL, USA) (Sabath and Denker, 2006). Madin-Darby Canine Kidney (MDCK) cells (CCL-34; ATCC, Manassas, VA, USA) were cultured on the filters in Eagle's Minimum Essential Medium supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin mix (Gibco®|Life Technologies, Grand Island, NY, USA).

Tissue Processing

Untreated and Cdt-treated HGX were fixed in 4% buffered formalin for paraffin sectioning at the University of Pennsylvania School of Dental Medicine Tissue Processing Facility. Sections were stained with hematoxylin and eosin and examined by light microscopy. Additional sections were pro-cessed for immunohistochemistry as described in the Fig. legend for each experiment. Stained slides were preserved with ProLong Gold anti-fade reagent containing DAPI (Life Technologies) and viewed with a Nikon Eclipse 80i fluores-cence microscope. Digital images were recorded with Spot Advanced 4.6 software (Diagnostic Instruments, Inc., Sterling Heights, MI, USA).
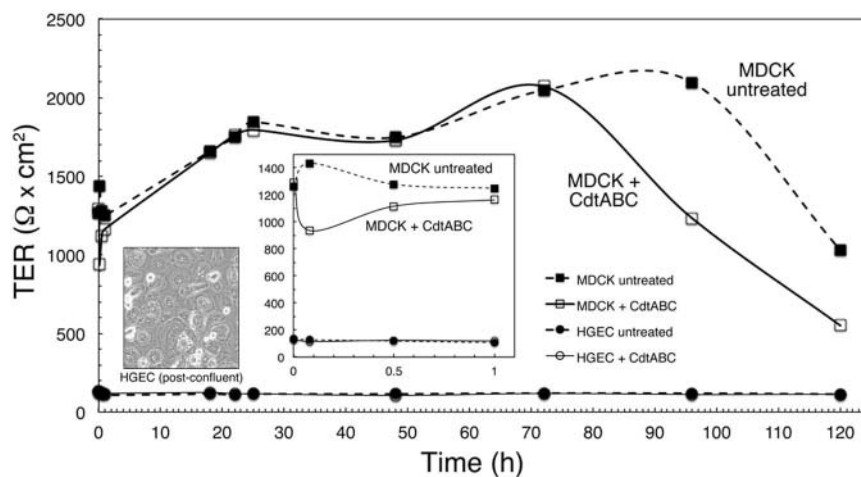
Figure 1. Transepithelial resistance (TER) of HGEC. HGEC cultured on polycarbonate filters were untreated and exposed to CdtABC for 18 hrs. No increase in the TER of either culture was detected over a 120-hour period. Cultures of untreated and CdtABC-treated MDCK cells reached a TER of approximately 1,800 Ω x cm² by 30 hrs of incubation. This experiment was performed twice.

Real-time (RT)-PCR

HGX were homogenized in TRIzol reagent (Life Technologies). Total RNA was isolated according to the manufacturer's proto-col, and a 2-µg quantity was converted into cDNA by means of the First-Strand cDNA Synthesis Kit with NotI-(dT)$_{18}$ primers (GE Healthcare, Piscataway, NJ, USA). RT-PCR reactions were performed in an ABI 7300 Real-Time PCR System with Power SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA, USA). Forward/reverse primers (5′ to 3′) [GAAGG TGACAGAGCCTCTGGA/GACCCCCTCCACAAATTGC (CDH1, E-cadherin); GATACCCAGCGCCGTACGT/GACCC CCTCCACAAATTGC (CTNNB1, β-catenin); GCATGGAGT CCTGTGGCAT/CTAGAAGCATTTGCGGTGGAC (ACTB, β-Actin)] were designed with Primer Express software (Applied Biosystems). TATA binding protein (TBP) mRNA expression level was used as a normalizing control. Results were expressed as relative fold change by the ΔΔCt method as described previ-ously (Damek-Poprawa *et al*., 2011).

RESULTS

HGEC Do Not Form Tight Junctions *in vitro*

TER was used to measure tight junctions in cultures of HGEC. MDCK cells, a commonly used positive control for tight junctions (Dukes *et al*., 2011), had resistance values as high as 2,000 Ω x cm² (Fig. 1). In contrast, TER for HGEC averaged 100 Ω x cm². Claudin 1, a component of tight junction filaments (Furuse *et al*., 2002), was poorly detected in both HGEC and HGX (data not shown). Therefore, the presence of tight junctions was not clearly evident. When the MDCK cells with established tight junctions were exposed to 10 µg/mL of Cdt, a slight decrease in the TER was observed within minutes. The resistance decreased by approxi-mately 80% by 72 hrs post-treatment. Untreated MDCK cells maintained a high TER for up to 100 hrs after reaching confluence.
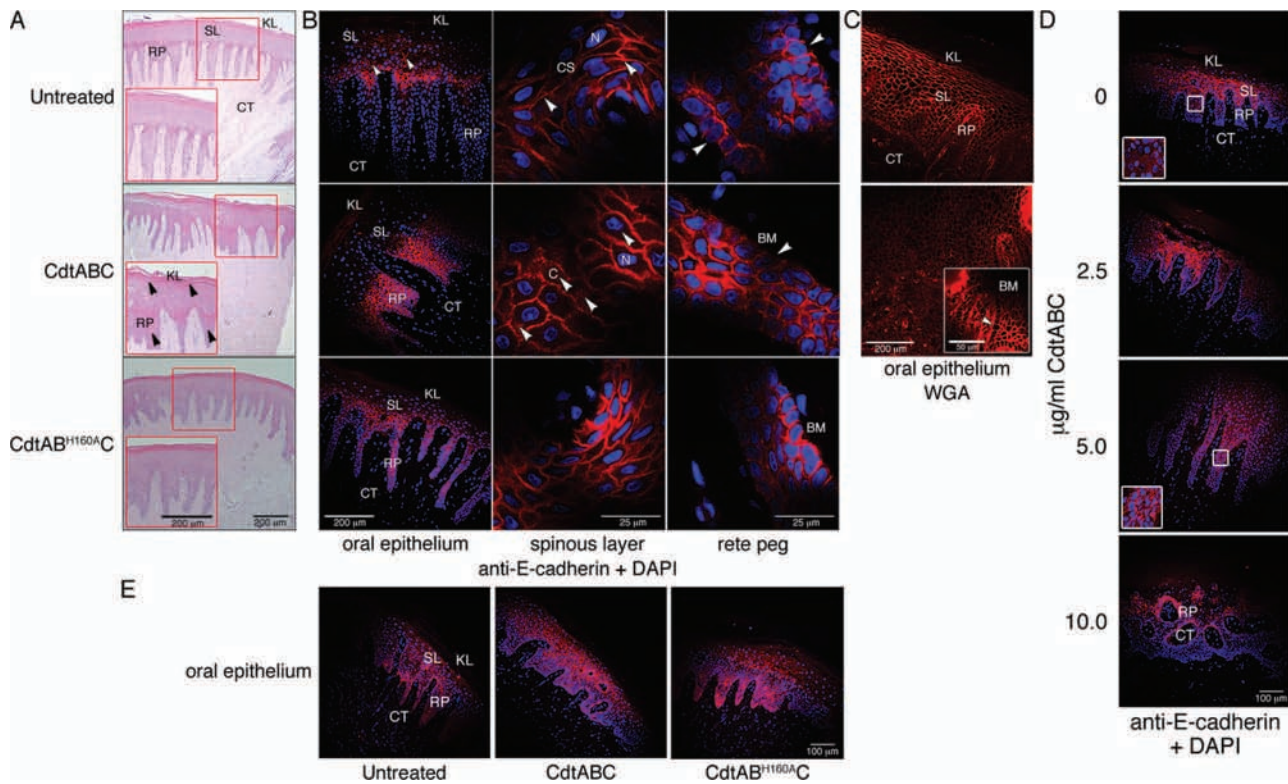
Figure 2. Cellular distribution of E-cadherin in untreated and Cdt-treated HGX. **(A)** Hematoxylin and eosin-stained sections of HGX that were untreated or treated with either 10 μg/mL CdtABC or CdtAB[H160A]C for 18 hrs. Arrows show the location of tissue damage. **(B)** Staining untreated and Cdt-treated (10 μg/mL for 18 hrs) HGX for the presence of E-cadherin. Arrows show the location of E-cadherin on the epithelial cell surface (CS), in the cytosol (C), and lack of staining at the junction of basal epithelial cells and the basement membrane (BM). **(C)** Untreated and Cdt-treated HGX labeled with 1:1,000 dilution of wheat germ agglutinin Alexa Fluor 555 conjugate (Life Technologies). Inset shows rete pegs (arrow) from CdtABC-treated HGX at a higher magnification. **(D)** Dose response of CdtABC-treated HGX stained for the presence of E-cadherin after 18 hrs of exposure. Insets show enlargement of outlined areas. The orientation of the tissue section in the bottom panel is showing a cross-section of the rete pegs. **(E)** E-cadherin distribution in the spinous layer of Cdt-treated gingival tissue. HGX sections shown in B, D, and E were labeled with a 1:100 dilution of rabbit polyclonal anti-E-cadherin IgG (Santa Cruz Biotechnology, Santa Cruz, CA, USA) and 5 μg/mL of conjugated goat anti-rabbit IgG Alexa Fluor 594 (red fluorescence; Life Technologies) and DAPI (blue fluorescence). KL, keratinized layer; SL, spinous layer; RP, rete peg; CT, connective tissue. Tissue from the same donor is shown in A through C, while those in D and E are from two distinct donors. Experiments were performed a minimum of 3 times with tissue from different patients.

Effect of Cdt on Cellular Levels and Distribution of E-cadherin

HGX from healthy patients were either left untreated or exposed to 10 μg/mL of wild-type CdtABC or CdtAB[H160A]C for 18 hrs. Hematoxylin and eosin staining of untreated HGX confirmed that the tissue was stable for the duration of the experiment. Rete pegs remained intact, cells in the spinous layer were densely packed, and the keratinized layer was undamaged (Fig. 2A). Upon exposure to CdtABC, there was extensive separation of the keratinized surface layer at the junction of the oral epithelium, as well as extensive disruption of the spinous epithelial layer and rete pegs. Structural integrity of HGX incubated with CdtAB[H160A]C appeared similar to that of the untreated tissue.

E-cadherin localized predominantly at the plasma membrane, outlining the epithelial cell-cell contact zone, in untreated HGX (Fig. 2B, top row). Exposure to CdtABC resulted in a pronounced increase in fluorescence intensity, thus representing an increase in the relative amount of E-cadherin. The protein was detected throughout the cytosol, indicating extensive intracellular redistribution (Fig. 2B, middle row). Lack of E-cadherin staining in untreated HGX suggested the absence of adherens junctions at the border of the epithelial cells and basement membrane. The presence of intact epithelial cell membranes adjacent to the basement membrane in all samples was confirmed by staining with wheat germ agglutinin (Fig. 2C). E-cadherin was detected circumferentially around cells adjacent to the basement membrane in HGX treated with CdtABC. Not only did this observation confirm that Cdt induces redistribution of E-cadherin within the epithelial cells, but it also suggests that the toxin causes detachment of basal cells from the basement membrane. Exposure of HGX to CdtAB[H160A]C affected the level of E-cadherin but not to the extent of that observed in HGX incubated with wild-type toxin (Fig. 2B, bottom row). The Cdt-induced change in distribution of E-cadherin was evident when the HGX were treated with between 2.5 and 5.0 μg/mL of the toxin (Fig. 2D).
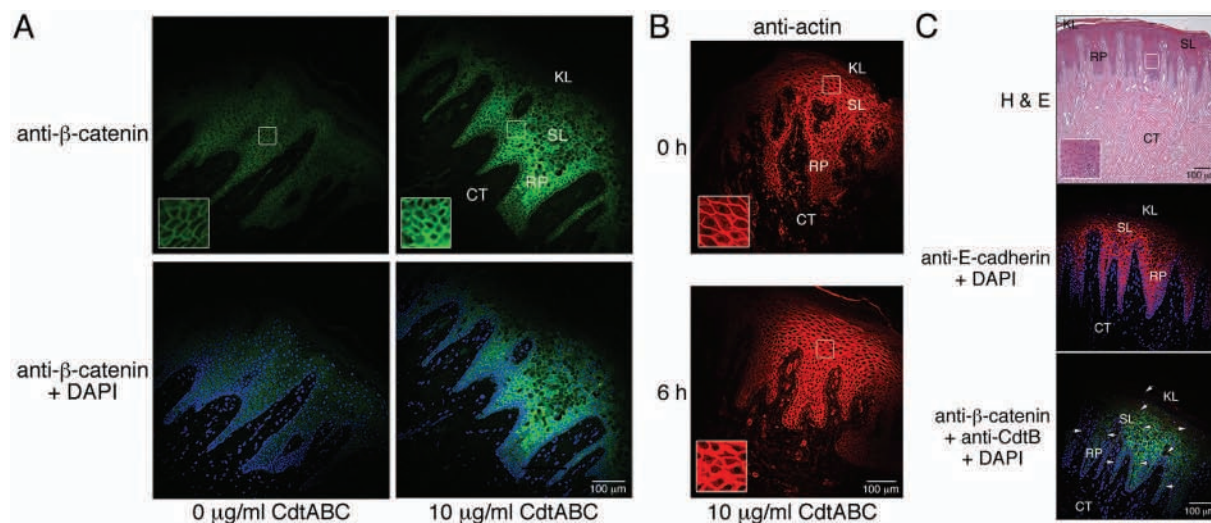
Figure 3. Localization of cell junction and structural proteins in untreated and CdtABC-treated HGX and gingival tissue from an individual with clinical signs of plaque-induced periodontal disease (PIPD).. **(A)** Sections from a HGX obtained from a healthy individual were treated with 0 or 10 μg/mL of CdtABC for 18 hrs and then were labeled with a 1:100 dilution of mouse monoclonal anti-β-catenin IgG (Santa Cruz Biotechnology) followed by 5 μg/mL of conjugated goat anti-mouse Alexa Fluor 488 (green fluorescence; Life Technologies). Sections were co-stained with DAPI. Outlined areas are enlarged in the insets. **(B)** Sections from HGX obtained from a healthy individual were treated with 10 μg/mL of CdtABC for 0 and 6 hrs and were labeled with a 1:100 dilution of rabbit polyclonal anti-actin IgG (Santa Cruz Biotechnology) followed by 5 μg/mL of conjugated goat anti-rabbit IgG Alexa Fluor 594 (Life Technologies). **(C)** Untreated HGX from an individual with clinical signs of disease. Sections were stained with hematoxylin and eosin and were co-labeled with anti-E-cadherin IgG, anti-β-catenin IgG, anti-CdtB IgG (red fluorescence in bottom panel), and DAPI. Antibody concentrations were the same as those used in Figs. 2 and 3A. The presence of CdtB (arrows) was detected with rabbit polyclonal anti-CdtB IgG (1:50,000 dilution) and 5 μg/mL of conjugated goat anti-mouse Alexa Fluor 488 as described previously (Damek-Poprawa *et al.*, 2011). The explants shown in A and B are from two separate healthy donors. Experiments were performed on healthy and diseased tissue a minimum of 3 and 2 times, respectively, with tissue from different patients.

Comparison of tissue from 14 different individuals revealed 2 distinct E-cadherin labeling patterns (9 spinous layer and 5 rete pegs), suggesting that distribution within the gingival epithelium may be patient-specific. As exemplified in Figs. 2B and 2D, the strongest reaction with the anti-E-cadherin antibody in untreated HGX was observed in the spinous layers. Upon exposure to increasing concentrations of CdtABC (Fig. 2D), the intensity of the signal within the basal epithelial cells increased. However, in HGX from other individuals, the staining pattern was reversed such that more intense labeling was apparent in the spinous cell layer after exposure of the HGX to the Cdt (Fig. 2E).

Effect of Cdt on Intracellular Scaffolding Proteins

Since adherens junction structure relies on dynamic interactions of E-cadherin with catenin complexes and the actin cytoskeleton (Drees *et al.*, 2005; Yamada *et al.*, 2005), we also examined the localization and relative levels of β-catenin and β-actin in the gingival epithelium of untreated and Cdt-treated HGX. Both proteins were detected in all tissue sections tested. Following 18 hrs of incubation with 10 μg/mL of CdtABC, the β-catenin concentration in the spinous layer was elevated relative to that in untreated HGX (Fig. 3A). Under similar treatment, there was a marked increase in actin concentration as early as 6 hrs after the addition of the Cdt (Fig. 3B).

Hematoxylin and eosin-stained sections of untreated HGX from patients with clinical signs of periodontal disease showed extensive tissue damage marked by detachment of the keratinized epithelium and disruption of the spinous layer and rete pegs

(Fig. 3C). Relatively high levels of E-cadherin and β-catenin were present in the spinous layer. Even though the tissue examined was from a diseased donor, it was surprising to detect anti-CdtB-positive deposits throughout the oral epithelium (red fluorescence in bottom panel).

Quantitative Evaluation of Junction Protein Gene Expression in HGX and HGEC

In response to the toxin, a statistically significant increase in E-cadherin mRNA expression level was detected in at least 50% of HGX and 66% of HGEC cultures from independent individuals (Fig. 4). Twenty-five percent of HGX and 50% of HGEC cultures exhibited a statistically significant increase in β-catenin expression. Actin expression was increased in 63% of HGX and 83% of HGEC cultures.

DISCUSSION

Recently, we showed that the Cdt from *A. actinomycetemcomitans* significantly altered the structure of the epithelial compartment of HGX (Damek-Poprawa *et al.*, 2011). In this study, we assessed the effects of the Cdt on adherens junctions, complexes that maintain tissue integrity under physiological conditions.

Our attempts to identify tight junctions, utilizing TER and immunolabeling for claudin-1, in post-confluent HGEC cultures were unsuccessful. The occurrence of tight junctions in human gingival epithelium had been suggested previously but not
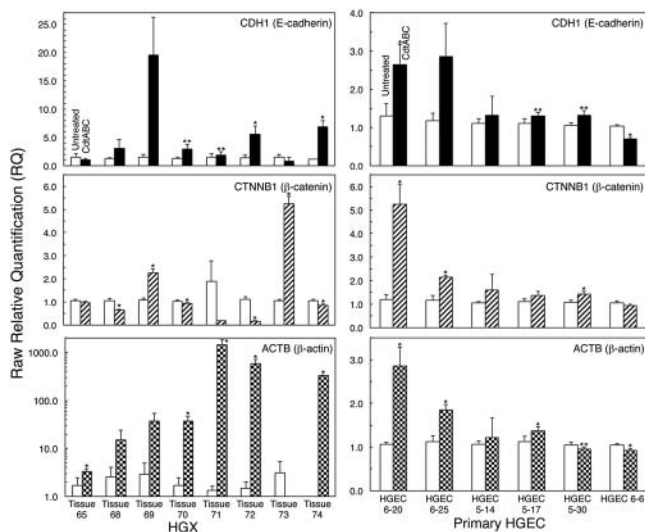
Figure 4. Quantitative expression of genetic markers for adherens junction and intracellular scaffolding proteins. RT-PCR of human genes for E-cadherin (CDH1), β-catenin (CTNNB1), and β-actin (ACTB) in untreated and CdtABC-treated HGX (eight individuals) and primary HGEC (six individuals). Untreated, white bars; CdtABC-treated (10 μg/mL), black and patterned bars. Note that the data are plotted with difference scales on the Y-axes. Mean values ± SD are shown (n = 3 in each group). Asterisks mark statistically significant differences (two-sample unequal variance test) between the relative expression levels of mRNA from untreated and CdtABC-treated HGX or HGEC ($*p \leq .05$, $**p \leq .1$). Experiments were performed a minimum of 3 times.

unequivocally demonstrated (Thilander and Bloom, 1968; Barnett and Szabo, 1973). Meyle and co-workers (1999) provided microscopic evidence of tight junctions in human gingival biopsies. However, they also indicated that there were areas in the tissue where tight junctions were not detected. The increase in TER of cultured human gingival keratinocytes was approximately 10 times lower than that of MDCK cells and was highly variable. The TER of gingival epithelial cell multilayers was only 150 Ω x cm^2 and not altered by exposure of the cells to either *Porphyromonas gingivalis* or *A. actinomycetemcomitans* for up to 24 hrs (Dickinson *et al.*, 2011). Based on these findings, and on data obtained *ex vivo*, it is possible that tight junctions may not be critical to maintaining the integrity of gingival epithelium.

Adherens junctions, composed of dynamic cadherin-catenin-actin complexes, are the most well-studied cell-cell adhesion complex in epithelial cells (Wheelock and Knudsen, 1991; Cavey and Lecuit, 2009; Meng and Takeichi, 2009; Brieher and Yap, 2012). We evaluated the distribution of E-cadherin and β-catenin in HGX. Relatively high concentrations of both proteins were detected in juxtaposition with the membrane of oral epithelial cells in untreated HGX. Interestingly, the lack of E-cadherin at the border between epithelial cells and basement membrane indicated the absence of adherens junctions in this region. This is most likely due to the fact that these cells attach to basement membrane *via* hemidesmosomes. These results supported previous observations that normal oral epithelium showed strong pericellular staining of E-cadherin in the basal and supra-basal cell layers but not on the basal aspect of keratinocytes adjacent to the basement membrane (Downer and Speight,

1993). Ye *et al.* (2000) also found marked expression of E-cadherin in the basal cell layer of healthy gingiva. With these findings at hand, we were confident that the HGX model would allow us to evaluate the effect of the Cdt on adherens junctions.

Our results indicated that the Cdt induces significant changes in both the distribution and expression of the component proteins of adherens junctions. Exposure of the HGX to wild-type Cdt resulted in a subtle but clearly detectable alteration in the cytosolic distribution of E-cadherin. Based on RT-PCR analysis, there was also an increase in expression of genes encoding E-cadherin, β-catenin, and β-actin in both HGEC and HGX from a majority of the donors examined. These effects may be associated with the cellular distension caused by the toxin. The variability that was detected in the response to the Cdt could be due to a multitude of environmental and/or donor-specific factors. Perturbations in the regulation of cell-cell junction stability and dynamics affect the barrier properties of epithelial layers and can have detrimental effects on tissue remodeling throughout development (Cavey and Lecuit, 2009). Therefore, barrier function is most likely a consequence of the stability of adherens junctions.

Considering the effect of Cdt on epithelial barrier function, we propose a model to explain these unique clinical findings. The clinical presentation of patients with LAP is typified by rapidly progressing bone loss despite a relative paucity of calculus and gingival inflammation. Secreted Cdt disrupts the gingival epithelium particularly, but not exclusively, by altering cell-cell contact. The bacterium and/or secreted products gain entry into the underlying connective tissue. Leukotoxin, another *A. actinomycetemcomitans* cytotoxin, induces cell death in a variety of inflammatory cells, thereby down-modulating the extent of clinically detectable gingival inflammation. The localized immunosuppression that results allows for additional influx of Gram-negative bacteria inhabiting the periodontal pocket. Lipopolysaccharide, a potent inducer of osteoclastogenesis, produced by the mixed infection causes the extensive bone loss seen in affected sites. The central premise of this model is that *A. actinomycetemcomitans* plays an early role in the pathogenesis of the disease *via* the elaboration of virulence factors that abrogate the host response. It is quite feasible that other organisms play a more critical role in latter events that directly lead to clinical manifestations of the disease.

ACKNOWLEDGMENTS

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