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ABSTRACT

Gingival overgrowth tissues have thickened connective tissue stroma, sometimes accompanied by the increased presence of collagen fibers, thickened epithelia, and elongated rete pegs. We have previously shown that expression of CCN2, also known as connective tissue growth factor (CTGF), correlates positively with the degree of gingival fibrosis, and that markers of epithelial to mesenchymal transition (EMT) are characteristic of all drug-induced forms of gingival overgrowth. Here we experimentally evaluate whether increased degradation of the basement membrane and apparent invasion of the underlying stroma by epithelial cells could be observed in human gingival overgrowth tissues. Tissues from 20 different individuals with human gingival overgrowth and 15 non-overgrowth samples were evaluated by histological analyses and by immunohistochemistry assays of basement membrane proteins. The results demonstrate that there are significantly higher numbers of basement membrane discontinuities in overgrowth tissues, sometimes containing epithelial-like cells. Disrupted basal membrane structure in gingival overgrowth tissues is accompanied by a discontinuous collagen type IV expression pattern and decreased laminin 5. These findings provide new additional support for the hypothesis that epithelial plasticity and EMT promote gingival overgrowth, resulting in compromised basal membrane structure and increased interactions between epithelial and connective tissue layers that contribute to fibrotic pathology.

KEY WORDS: gingival overgrowth, fibrosis, basement membrane.

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Loss of Basement Membrane Integrity in Human Gingival Overgrowth

INTRODUCTION

Enlargement of gingival tissues is an adverse side-effect of certain chemotherapeutic agents (Trackman and Kantarci, 2004). The reported incidence of gingival overgrowth could be as high as 70% in response to certain drugs such as the anti-seizure medication phenytoin (Hassell, 1981), emphasizing the severity of this clinical problem. In addition to being disfiguring, gingival overgrowth leads to poor oral hygiene and debilitated oral function in individuals already suffering from major diseases and conditions such as epilepsy and cardiovascular diseases, or immunosuppression in patients medicated with phenytoin, calcium channel blockers, or cyclosporin A (Li *et al.*, 2000). Histopathological studies have demonstrated that gingival tissues are abnormal in several respects (Trackman and Kantarci, 2004). In addition to fibrosis in the lamina propria, drug-induced gingival overgrowth is associated with thickening of epithelium and elongated rete pegs extending deep into the underlying connective tissue (Pernu *et al.*, 1989). This phenomenon is related to expansion of the *Stratum spinosum* and is associated with increased mitotic activity within the epithelial layer (Ayanoglou and Lesty, 1999; Saito *et al.*, 1999; Sume *et al.*, 2010). It is not known, however, how such epithelial structures form and function in gingival overgrowth tissues. The underlying hypothesis of this and a previous report is that elongated rete pegs in gingival overgrowth may result from an increased level of epithelial plasticity, resulting in mesenchymal characteristics that can be considered to be a phenotypic transition known as 'epithelial to mesenchymal transition' (EMT) (Sume *et al.*, 2010).

CCN2, also known as connective tissue growth factor (CTGF), and TGF- β 1 regulate collagen turnover in gingival connective tissues. TGF- β 1 is secreted by a variety of cell types and up-regulates the expression of CCN2 (Duncan *et al.*, 1999; Hong *et al.*, 1999). CCN2, in turn, stimulates collagen accumulation and deposition by human gingival fibroblasts (Heng *et al.*, 2006). We have previously shown that CCN2 expression correlated positively with gingival fibrosis where phenytoin-induced gingival overgrowth specimens have the highest levels of both CCN2 and fibrosis (Uzel *et al.*, 2001; Kantarci *et al.*, 2006). In addition, not only is CCN2 highly expressed in connective tissue cells and matrix, but elevated CCN2 levels were also seen in gingival epithelial cells in fibrotic tissues, suggesting that CCN2 plays a role in both epithelial cell proliferation and in increased accumulation of collagen deposition and fibrosis in the connective tissue stroma (Kantarci *et al.*, 2006).

We have recently reported that gingival overgrowth tissues express some markers of EMT, and that cultured primary human gingival epithelial cells can be induced to undergo changes consistent with EMT *in vitro* by TGF- β 1 treatment. EMT is a process in which the phenotypic plasticity of epithelial

cells results in greater mesenchymal characteristics. In particular, polarity and cell-cell contacts between epithelial cells are lost, and epithelial cells become motile, migrate into connective tissue stroma, and integrate into connective tissue (Sume *et al.*, 2010). Although additional mechanisms of fibrosis clearly exist (Krenning *et al.*, 2010), the biological process of EMT predicts that, for epithelial cells to migrate into the connective tissue stroma, some degree of degradation of the basement membrane must occur. Here, we experimentally investigate whether evidence for an increased level of compromised basement membrane structures can be observed in human gingival overgrowth tissue samples to provide additional evidence for a functional contribution of an EMT-like process to gingival overgrowth. Analysis of the data indicated that gingival basement membranes in all drug-induced forms of gingival overgrowth are disrupted, and that discontinuities are significantly increased in histological stains, and in immunohistochemistry analyses for collagen type IV and laminin 5.

MATERIALS & METHODS

Chemicals and Reagents

Goat antihuman type IV collagen polyclonal antibody was purchased from Millipore (Billerica, MA, USA), and mouse antihuman epiligrin (laminin 5) monoclonal antibody was purchased from Chemicon (Temecula, CA, USA). Dulbecco's modified phosphate-buffered saline (PBS) was purchased from Life Technologies (Grand Island, NY, USA). All other reagents were purchased from Sigma Aldrich (St. Louis, MO, USA).

Gingival Tissues

Gingival tissue samples were obtained from individuals undergoing periodontal surgery in the Department of Periodontology and Oral Biology and the Clinical Research Center of Boston University at the Goldman School of Dental Medicine and the Franciscan Children's Hospital and Rehabilitation Center. Samples from 35 donors were included in this study: phenytoin-induced gingival overgrowth, $n = 6$; cyclosporine-A-induced, $n = 6$; nifedipine-induced, $n = 8$; and control tissues from systemically healthy donors without gingival overgrowth, $n = 15$. Written informed consent from all donors was obtained with the approval of the Institutional Review Board of Boston University Medical Center. All participants in this study were 20 years of age and older. Age, gender, and clinical inflammation (gingival index and bleeding on probing) were recorded for each individual immediately before surgical procedures. Gingivectomy and other periodontal surgical procedures were performed after initial periodontal treatment, including professional elimination of supra- and subgingival plaque and maintenance of proper oral hygiene.

Tissue Fixation and Sectioning

Tissues were fixed in 4% paraformaldehyde in phosphate-buffered saline (PBS) at 4°C for 4 hrs and were incubated in 30%

sucrose overnight. They were then placed in 2-methylbutane at -80°C . At least 20 serial 5- μm frozen sections were made for each tissue sample on a cryostat.

Immunohistochemistry

Immunohistochemistry of human gingival tissue samples was performed with the use of a temperature-controlled staining system to standardize staining conditions and visualized with Elite Vecta Stain Kits (Vector Laboratories, Burlingame, CA, USA), as previously described (Uzel *et al.*, 2001). Working concentrations for primary antibodies were 10 $\mu\text{g}/\text{mL}$ for type IV collagen and 10 $\mu\text{g}/\text{mL}$ for laminin 5. Serial sections were stained with non-immune IgG (10 $\mu\text{g}/\text{mL}$).

Evaluation

The orientation of each sample was determined at 100x magnification as follows. We were careful to observe the sulcular epithelium, oral epithelium, and connective tissue in the same section with no folding where epithelial-connective tissue distinction was clear. During this initial inspection, we also assessed planar orientation of epithelial cells, eliminating the possibility for artifactual appearances in the lengths of the rete pegs and other epithelial structures. Sections in which epithelial cells did not appear flat and planar throughout were not used. We also used the epithelium to orient the tissue anatomy by identifying and distinguishing the oral epithelium and sulcular epithelium based on their distinct histological characteristics (*e.g.*, lack of the keratinized layer in sulcular epithelium). Tissue sections that did not meet these strict criteria were not used for analyses, and original fixed tissues were re-sectioned to provide correctly oriented sections, as needed.

From 3 to 5 serial sections from each individual were evaluated at 5 different locations *per* section. We were careful to evaluate only sections with clear and intact epithelium-connective tissue interfaces. The following criteria were then applied to suboral and subsulcular tissue sites. All investigators were blinded to the identity of samples and calibrated regarding features of interest in tissue samples. Each investigator was trained regarding the histological structures of gingival tissues. Each slide was evaluated at 2 different times by three different individuals. First evaluations were made by ZN, Y-SK, and SSS, and results were transferred to an Excel spreadsheet. A second round of blinded measurements was made by AK. Results were compared, and inconsistencies were resolved by re-measurement prior to investigator un-blinding. Initial observations and confirmation of disruptions in basal lamina structure were made at 200x magnification. Quantification was done by computer-assisted image analysis at 1000x magnification (Image-pro plus 4.0, Media Cybernetics, Bethesda, MD, USA). Lengths of basal lamina were measured with the use of a computerized ruler, and numbers of disruptions of the basal lamina were counted. Degrees of disruption were categorized as "1" if a "break" in the continuity of the lamina occurred and as "2" if the break was accompanied by cells resembling epithelial cells or fibroblasts located in the break itself. The total numbers of breaks

Figure 1. Basal lamina structure in gingival biopsies from normal control tissues and overgrowth tissues. **(A)** Hematoxylin-and-eosin-stained control and phenytoin gingival overgrowth specimens at low magnification (100x), showing elongated rete pegs and irregularities (arrows) at the epithelium/stroma interface (bar = 400 μ m). **(B)** Representative images of hematoxylin-and-eosin-stained specimens, showing no overgrowth control, and drug-induced gingival overgrowth specimens (control, CON; phenytoin, PHE; nifedipine, NIF; cyclosporin-A, CSA). Epithelium (Ep) of fibrotic lesions shows deep extensions (rete pegs, white arrow) into underlying connective tissue stroma (Ct). The continuity of the basal lamina of the epithelium (shown by the black arrows) is disrupted in samples from overgrowth tissues (red arrows). Such disruptions ("breaks") are sometimes associated with areas of increased inflammation (shown by *). Measure bars are 40 μ m long at a magnification of 1000x.

were calculated and normalized to the length of basal lamina for measurement of the density of the breaks. In parallel, the degree of inflammation was measured by the number of inflammatory cells *per* area as described (Uzel *et al.*, 2001). Inflammatory indices of tissues adjacent to the subsulcular epithelia in proximity to the tooth surfaces were compared with the suboral epithelium distant to the tooth surfaces in gingival overgrowth and non-overgrowth control tissues (Sume *et al.*, 2010). The degree of continuity of the collagen IV and laminin 5 immunohistochemical staining was characterized.

Statistical Analysis

Results were expressed as mean $\pm$ standard error of the mean for each evaluated site. For statistical analysis, ANOVA with Bonferroni correction was used for the comparison of differences. Significance was declared if $p < 0.05$.

RESULTS

Basement Membrane Histology

Histology of gingival samples from individuals with drug-induced gingival overgrowth showed hyperplasia with dense, elongated rete pegs extending deep into connective tissues. Enlarged rete pegs and irregularities at or near the basement membrane were prevalent in phenytoin-induced gingival overgrowth specimens (Fig. 1A), suggesting that the fibrotic transformation is accompanied by increased extensions of the epithelia into stroma. To study these alterations of the basement membrane more closely, we then studied the entirety of the epithelium-connective tissue interface at higher magnification. All specimens from the phenytoin gingival overgrowth individuals showed evidence of disruptions and discontinuities in the basal membrane (Fig. 1B). Many of these "breaks" in the basal membrane were accompanied by the presence of cells that we speculate may have migrated from the epithelium.

Figure 2. Quantitative analysis of samples with basal lamina disruptions. (A) Percentage of samples with disruptions in basal lamina is presented. (B) Samples were compared in areas with or without inflammatory infiltration. In the human gingiva, inflamed sites are typically located below the gingival crevice (sulcus) at the interface of the tooth and the gingival attachment. Subsulcular areas demonstrate more obvious inflammatory infiltrates, whereas the soft tissues distant to the tooth surfaces are areas with less inflammation. Mean $\pm$ standard error of the mean values are shown. ANOVA with Bonferroni correction; * $p < 0.05$ compared with controls; ** $p < 0.01$ compared with controls. CON, $n = 15$; PHE, $n = 6$; NIF, $n = 8$; CSA, $n = 6$. GO represents the entire gingival fibrosis group of individuals studied ($n = 35$).

Quantitative analyses indicated that the number of gingival overgrowth tissues with disruptions in basal lamina was significantly higher compared with that in the non-overgrowth control specimens (Fig. 2A). Nearly all phenytoin-induced fibrosis samples contained disruptions, and levels were significantly higher compared with those in controls ($p < 0.01$). Samples from individuals with nifedipine-induced gingival overgrowth also contained higher numbers of discontinuities compared with those from control individuals in both inflamed and less-inflamed regions ($p < 0.05$). By contrast, cyclosporine A samples contained an increased number of breaks compared with the control specimens only at non-inflamed suboral gingival sites, while inflamed subsulcular areas contained numbers of breaks that were no different from those in inflamed controls (Fig. 2B). Tissues from phenytoin and nifedipine individuals contained significantly more breaks at inflamed sites, while phenytoin tissues contained significantly more breaks in all areas compared with those from all other groups. Subsulcular sites, which represent more inflamed areas, close to the dental roots and adjacent to the periodontal pocket, have more breaks compared with suboral areas in control specimens. There was no statistically significant difference found in breaks among the different gingival overgrowth groups. In contrast, non-overgrowth tissues contained fewer breaks in non-inflamed tissue areas compared with inflamed sites ($p < 0.05$),

suggesting that inflammation also contributes to disruptions of the basal lamina.

We next studied the number of breaks *per* tissue specimen, and classified breaks as either showing evidence of no apparent cell penetrations (degree 1), or breaks containing cells (degree 2). In addition, we analyzed the density of different types of breaks (breaks/mm basement membrane) to determine the severity of the basal lamina disruption in each group. Gingival overgrowth specimens overall contained increased total numbers of breaks and an increased number of second degree breaks with epithelial-like cell infiltrations compared with non-overgrowth gingival tissues (Fig. 3A). Fig. 3B presents the density of the disruptions where the total numbers of breaks were normalized to the length of the basal lamina. Analysis of the data, taken together, suggests that phenytoin and nifedipine gingival overgrowth tissues contain the most severe disruption of the basal lamina, with significantly higher numbers and severity

seen in all parameters measured compared with control tissues ($p < 0.05$).

Collagen Type IV and Laminin 5 Expression in Human Gingival Basement Membranes

Next, we studied the immunohistochemical staining patterns of collagen type IV, the major basement membrane collagen, and laminin 5, an important adhesive glycoprotein component of basement membranes. Fig. 4 demonstrates that expression of collagen type IV is continuous and without breaks in control tissues. All forms of gingival overgrowth specimens demonstrated a pattern of breaks and discontinuities of collagen type IV expression compared with the control samples. Continuous laminin 5 staining was similarly observed in control samples, while all forms of gingival overgrowth appeared to have a thinner and more diffuse pattern of laminin 5 staining with disruptions in the continuity of the basement membrane.

DISCUSSION

In this study, we have investigated the integrity of the basement membranes in specimens from individuals with gingival overgrowth resulting from long-term medical therapy with phenytoin,

nifedipine, or cyclosporin A. Our previous studies have provided evidence that fibrotic enlargement of the gingival tissue is regulated by TGF- β 1 and CCN2 and an imbalance of inflammatory mediators, accompanied by markers of EMT (Uzel *et al.*, 2001; Kantarci *et al.*, 2006; Sume *et al.*, 2010). Elongated epithelial structures extending deep into the underlying connective tissues are characteristic of gingival overgrowth (Pernu *et al.*, 1989; Seymour *et al.*, 1996; Ayanoglou and Lesty, 1999). This histopathology suggested to us that EMT could be a biological process contributing to gingival overgrowth in which epithelial cells lose cell-to-cell contacts and become motile and migrate into the underlying connective tissue stroma. Evidence for the loss of E-cadherin and gain of mesenchymal markers in gingival overgrowth epithelial layers and gain of mesenchymal markers has been previously reported (Sume *et al.*, 2010). Because compromised basement membrane integrity is an important feature of EMT, we here investi-

gated whether evidence for gingival basement membrane structural defects could be observed in clinical human samples of gingival overgrowth compared with control tissues. The findings now presented demonstrate that disruptions in basement membranes are more frequent and severe in gingival overgrowth, particularly in phenytoin-induced gingival overgrowth, which is the most fibrotic form of this drug-induced pathology. Increased inflammation is associated with disruption of the basement membrane structures in non-overgrowth tissues, while drug-induced overgrowth tissues have disrupted basement membrane structures whether or not an abundance of inflammatory cells was observed. This suggests that cytokine balances may be altered and stimulate EMT in gingival overgrowth, even in the absence of an exaggerated inflammatory response. Meanwhile, expression of collagen IV and laminin 5 protein staining patterns is consistent with histologic analyses and points to the importance of assessing for regulation of basement membrane structural turnover in animal models of gingival overgrowth for a better understanding of the dynamic aspects of gingival overgrowth development. EMT is typically accompanied by increased expression and activation of proteolytic enzymes including MMPs, as previously noted (Sume *et al.*, 2010).

Previous studies from our laboratory have identified CCN2 to be strongly induced by TGF- β 1 in gingival fibroblast cultures, and CCN2 is highly expressed *in vivo* in connective tissue

Figure 3. Structural analyses of the basal lamina. Each measurement was repeated on 5 sections per specimen. **(A)** Disruptions in the basal lamina ("breaks") were classified as degree 1, with a clear and reproducible disruption in all 5 sections without the apparent presence of cells in the gap itself. When cells were observed in the connective tissue and in proximity to the breaks, this was classified as degree 2 disruption. The total number of breaks was calculated as a sum of degrees 1 and 2 disruptions. **(B)** The density of the breaks was measured as the total number of breaks per mm of the length of basal lamina. Mean $\pm$ standard error of the mean values is shown. ANOVA with Bonferroni correction; * $p < 0.05$ compared with controls. CON, $n = 15$; PHE, $n = 6$; NIF, $n = 8$; CSA, $n = 6$. GO represents the entire gingival fibrosis group of individuals studied ($n = 35$).

stroma of gingival overgrowth samples (Uzel *et al.*, 2001; Kantarci *et al.*, 2006). Moreover, CCN2 is expressed not only in the connective tissue stroma, but is also highly expressed in gingival epithelial cells *in vivo* in drug-induced human gingival fibrotic tissues, and not in normal tissues, suggesting that CCN2 has biological functions in epithelial cells. Since gingival overgrowth is characterized by increases in both epithelial and connective tissue layers, it is possible that CCN2 is playing a role in the proliferation of basal gingival epithelial cells. During EMT, we speculate that partial destruction of the basement membrane can lead to inappropriate diffusion of factors between the connective tissue and epithelial layer tissues, thereby sustaining CCN2 epithelial cell expression and proliferation.

In summary, analysis of the data indicated that drug-induced gingival overgrowth tissues exhibit marked discontinuities in basement membranes. These findings lend further experimental support to the notion that epithelial plasticity and EMT contribute to the development of drug-induced gingival overgrowth. Further insight into the molecular and cellular events that lead to gingival overgrowth and the application of potential therapeutic approaches already identified (Black *et al.*, 2007; Black and Trackman, 2008) awaits the application of animal models to address this disfiguring and problematic oral pathology.

Figure 4. Collagen type IV and laminin 5 expression in human gingival basal lamina, counterstained with hematoxylin. **(A)** Non-immune staining of the human gingival specimens demonstrates epithelium (Ep) and connective tissue (Ct) compartments of the human gingiva in overgrowth or non-overgrowth biopsies (control, CON; phenytoin, PHE; nifedipine, NIF; cyclosporin-A, CSA). No background staining was observed. Measure bars are 400 μ m at a magnification of 100x. **(B)** Collagen type IV and laminin 5 were stained by immunohistochemistry as described in MATERIALS & METHODS. Black arrows indicate the basal lamina as a continuous layer at the interface of the epithelium (Ep) and connective tissue (Ct) in non-overgrowth (CON) samples. Breaks are clearly observed in the basal lamina of the overgrowth samples (red arrows) where collagen IV expression is not observed. Collagen IV and laminin expression were decreased as the extensions of the epithelium were inserted into the connective tissue. Measure bars represent 40 μ m at a magnification of 1000x.

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