Chronic ulcerative stomatitis: evidence of autoimmune pathogenesis

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Objectives. Chronic ulcerative stomatitis is a condition characterized by chronic, painful oral ulcers, whose pathogenesis is unknown. Patients demonstrate specific IgG autoantibodies against ΔNp63α, an epithelial nuclear transcription factor. The aim of this study was to investigate the role of patient autoantibodies in the disease pathogenesis.

Methods. Three-dimensional in vitro tissues consisting of a fully differentiated, multilayer epithelium that mimics its in vivo counterpart were incubated with serum from patients with chronic ulcerative stomatitis.

Results. Our results show a subepithelial detachment of the epithelium at the basement membrane interface, mimicking the oral ulcerations that are seen clinically. Expression of basement membrane proteins Type IV collagen and laminin-5 was unaltered, whereas the expression of α6β4 integrins, hemidesmosome components that attach basal keratinocytes to the basement membrane, was reduced, as determined by immunohistochemistry.

Conclusion. These results give evidence that patient autoantibodies are pathogenic; and support an autoimmune pathogenesis in chronic ulcerative stomatitis. (Oral Surg Oral Med Oral Pathol Oral Radiol Endod 2011;111:742-748)

Chronic ulcerative stomatitis (CUS) is a poorly understood, chronic condition causing ulcerations, particularly in oral mucous membranes.1 Because the histologic features of CUS are not specific, diagnosis requires a biopsy with direct immunofluorescence examination, which detects patient autoantibodies in the specimen. The literature contains reports of 39 CUS cases;2 however, it is likely that many cases are not diagnosed because of the need for special processing and the high cost of direct immunofluorescence. Because the etiology is unknown, treatment of CUS is empiric. The most effective treatment involves off-label use of hydroxychloroquine, an antimalarial drug that provides relief in many cases but is not well-tolerated by some patients and may have serious side effects, such as retinopathy, toxic psychosis, neuromyopathy, agranulocytosis, and aplastic anemia.3 Understanding the pathogenesis of CUS may lead to a better pharmacologic therapy. The purpose of this study was to investigate the etiology of CUS by assessing the role of ΔNp63α-specific autoantibodies using a 3-dimensional (3D) in vitro model of fully stratified epithelium that closely mimics the architecture of the oral mucosa.

The diagnostic finding on direct immunofluorescence examination of a CUS patient biopsy is a speckled fluorescence in the nuclei of basal and parabasal epithelial cells known as a stratified epithelium specific–antinuclear antibody (SES-ANA) pattern.4 This pattern is generated because CUS patients have autoantibodies that bind to a specific protein, ΔNp63α, which is normally present in the nuclei of oral epithelial basal keratinocytes.5 ΔNp63α is an isoform of p63, which is a member of the p53 family of nuclear transcription factors. p63 acts as a master regulator and has pleiotropic effects on embryogenesis and limb morphogenesis, as well as on the phenotype and development of stratified squamous epithelia, adnexa, teeth, and glands.6-8 Several isoforms of p63 exist in which the N-terminus may be either transactivating (TA) or truncated (ΔN), depending on the promoter start site, and the C-terminus has either α, β, or γ variations owing to alternative splicing.9 The predominant p63 isoform expressed in oral epithelium is ΔNp63α.

Although sera from CUS patients contains autoantibodies to ΔNp63α, the role of these autoantibodies in the etiology of CUS remains unclear. Whether the autoantibodies to ΔNp63α in CUS patients are pathogenic antibodies (Abs) that are responsible for the clinical manifestations, or merely physiological autoantibodies, is unknown. We have used 3D human epithelial tissues that demonstrate the morphologic hallmarks of

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mucosal tissues as a surrogate model to study CUS pathogenesis. Our findings demonstrate proof-of-concept that 3D human epithelial tissues provide an important platform to the study of oral disease processes and support their role in future studies designed to test new therapeutic approaches to treat CUS. We demonstrate that serum from CUS patients with antibodies to ΔNp63α induce separation between the epithelium and connective tissues that mimic the loss of oral epithelium that manifests in this condition, confirming the hypothesized role of ΔNp63α-specific Abs in the pathogenesis of CUS.

MATERIAL AND METHODS

Cell cultures

Normal human keratinocytes (NHK) were established through trypsinization of newborn human foreskin fragments and grown on irradiated 3T3 fibroblasts in keratinocyte medium. Keratinocytes were maintained in Dulbecco’s Modified Eagle’s Medium (DMEM) with 10% fetal bovine serum. Human dermal fibroblasts were derived from foreskins and grown in DMEM with 10% fetal bovine serum (FBS). NHK and foreskin fibroblasts (HFFs) were expanded and banked before being used for construction of human skin equivalents (HSEs).

Construction of human skin equivalents

HSEs were created as organotypic cultures grown as collagen rafts. Briefly, early-passage HFFs were added to neutralized type-I collagen (Organogenesis, Canton, MA) to a final concentration of 2.5 × 10⁴ cells/mL. A 3-mL aliquot was added to each 35-mm well of a 6-well plate and incubated for 4 to 6 days in media containing DMEM and 10% FBS until the collagen showed no further shrinkage. NHKs (5 × 10⁵ cells) were seeded on the surface of the contracted collagen gel. Cultures were submerged in low-calcium epidermal growth medium for 2 days, in normal-calcium epidermal growth medium (EGM; Organogenesis) for 2 days, then raised to the air-liquid interface and fed from below with normal-calcium cornification medium for an additional 3 to 10 days. Cultures were performed in triplicate and maintained for 2, 9, and 14 days.

Serum samples and monoclonal p63 antibody

CUS sera were clinical samples from 4 patients whose sera were characterized in previous publications; all sera had immunoglobulin G (IgG) Abs to the N-terminal portion of the ΔNp63 protein by immunoblot and enzyme-linked immunosorbent assay (ELISA). Normal human serum (NHS) was obtained by venipuncture from a healthy, 53-year-old volunteer. All of the CUS sera were existing diagnostic samples and exempt from human subject review. Monoclonal p63 antibody (Ab) 4A4 (Santa Cruz Biotechnology, Santa Cruz, CA) was added at a concentration of 1.25 µg/mL. Experimental HSEs were treated with either CUS serum or monoclonal p63 Ab 4A4 for 48 hours. Negative controls were either not treated or treated with NHS. The volume of serum was calibrated to the ELISA results to ensure that the same concentration of autoantibodies was used from the different patient samples. Serum dilutions in culture media were approximately 1:10 or 1:100, designated as high and low concentrations, respectively.

Immunofluorescence and histology

HSEs were frozen and stored at −70°C. Tissues were processed by placement in 2 M sucrose for 2 hours at 4°C, and then frozen in Tissue-TEK optimal cutting temperature embedding media (Sakura Finetek, USA, Inc., Torrance, CA) in liquid-nitrogen vapors. Serial sections (6-µm) were cut on a cryostat (Microm, Waldorf, Germany), mounted onto gelatin-chrome albumen-coated slides, washed with phosphate-buffered saline (PBS), and blocked with 0.05% goat serum, and 0.2% bovine serum albumin (BSA), vol/vol in PBS without fixation. Sections were incubated with appropriate monoclonal antibodies, p63 Ab 4A4 (Santa Cruz Biotechnology), laminin 5 (D4BJ, Chemicon International, Inc., Temecula, CA), α6 integrin subunit (G0H3, Chemicon International, Inc.), β4 integrin (Millipore, Billerica, MA), and type IV collagen (Abcam, Cambridge, MA). Appropriate secondary antibodies were used, e.g., Alexa 594-conjugated, goat anti-rat, antimouse, or anti-human IgG (Molecular Probes, Eugene, OR). Slides were coverslipped with Vectashield containing 1 µg/mL DAPI (Vector Laboratories, Burlingame, CA). Fluorescence was visualized using a Nikon Eclipse 600 microscope (Japan), and photomicroscopy was performed using a Texas Red filter. For routine light microscopy, HSEs were fixed in 10% neutral buffered formalin, embedded in paraffin, and 4-µm sections were stained with hematoxylin and eosin.

RESULTS

Cus autoantibodies colocalize with ΔNp63α

HSEs incubated with and without CUS serum were stained with DAPI (blue), monoclonal p63 Ab 4A4 (red), and anti-human IgG Ab (green) to show that CUS patient Abs recognize ΔNp63α in HSEs. Untreated HSEs showed that basal keratinocytes contain nuclear p63 protein but do not demonstrate human antibodies in basal cell nuclei (Fig. 1, A). Incubation of HSEs with low CUS serum concentrations showed that IgG colocalized with ΔNp63α in the nuclei of some basal layer keratinocytes (Fig. 1, B).
Incubation of HSEs with high CUS serum concentrations showed the nuclear localization of IgG, which masks the signal from the monoclonal p63 antibody (Fig. 1, C). These results show that CUS patient autoantibodies bind to ΔNp63α in the HSE basal keratinocyte nuclei, which mimics the diagnostic findings in vivo seen upon direct immunofluorescence.

CUS autoantibodies and monoclonal p63 antibodies lead to epithelial detachment in 3D epithelial tissues

HSEs incubated with NHS show no change from the untreated controls (Fig. 2, A). HSEs treated with low CUS serum concentrations did not show changes in epithelial architecture and showed an intact basement membrane when compared with control HSEs (Fig. 2, B). HSEs treated with high CUS serum concentrations showed a subepithelial split at the epithelial-stromal interface and complete epithelial detachment in focal areas (Fig. 2, C). HSEs treated with a monoclonal Ab to ΔNp63α also showed complete epithelial detachment (Fig. 2, D). These results demonstrate that CUS serum Abs induce epithelial detachment, from the underlying stroma-basement membrane interface.

Basement membrane synthesis and intercellular adhesion was unaffected by CUS autoantibodies

HSEs incubated with and without CUS serum were stained by immunohistochemistry with antibodies directed against the basement membrane components type IV collagen (Fig. 3, A) and laminin-5. Serum-treated HSEs did not show aberrant synthesis of type IV collagen (Fig. 3, B) or laminin-5 (Fig. 3, C), in spite of the subepithelial split being present within the basement membrane.

Antibody-treated human skin equivalents show decreased expression of α6β4 integrins

HSEs incubated without CUS serum showed expression of α6β4 integrins on the basal surface of basal keratinocytes in a linear distribution at the basement membrane interface (Fig. 4, A). In contrast, HSEs treated with high CUS serum concentrations showed a reduced expression of α6β4 integrins, a patchy distribution of α6β4 integrins, and epithelial detachment (Fig. 4, B).

DISCUSSION

By using 3D human tissues that mimic many features of stratified squamous epithelium, our study is the first to determine that the pathogenesis of CUS is linked to disruption of the basement membrane interface by CUS autoantibodies. The CUS sera used in this study were from patients harboring autoantibodies directed to ΔNp63α as confirmed by immunoblot and ELISA in previous studies. Our results show that addition of CUS patient serum to an HSE model replicated the in
vivo localization of CUS autoantibodies within keratinocyte nuclei in the basal epithelial layer and produced epithelial detachment, mimicking epithelial ulceration, which is the hallmark of CUS. Addition of a monoclonal antibody to the 3D tissue model also produced epithelial detachment, which for the first time identifies that CUS autoantibodies to ΔNp63α are involved in CUS pathogenesis.

The integrity of stratified squamous epithelium depends on several factors, such as intercellular attachments between individual keratinocytes and attachment between basal keratinocytes and the basement membrane components. Defects in any of these components may result in clinical detachment and ulcer formation as illustrated by clinical diseases, such as pemphigus,
and controls expression of epithelial detachment. Some, which we postulate may be linked to the sub-
avasculature as a variety of cell-adhesion molecules, including laminin-binding integrins (α3, β1, α6, β4) and integrin subunits that bind to fibronectin, such as α5 and β1. Furthermore, it is known that p63 can drive expression of β4 integrin through AKT kinase activation. We suggest that antibody binding to ΔNp63α interferes with the DNA binding of this transcription factor resulting in dysregulation of α6β4 integrin gene expression and function. Previous epitope mapping studies showed that CUS autoantibodies bind to a folded configuration of the ΔNp63α DNA-binding domain, which supports the hypothesis of antibody-mediated interference with ΔNp63α function. p63 sequestration by mutant p53 protein has recently been shown to alter integrin regulation.

Autoimmune disease classification must meet the following specific criteria: (1) that an autoimmune response be in the form of an autoantibody or T-cell-mediated immunity, (2) that the corresponding antigen be identified, (3) that an analogous immune response be induced in an experimental animal, and (4) that an immunized animal must develop a similar disease.

Our results fulfill the first 2 of Witebsky et al.’s criteria; experimental animal studies have not been performed. Current immunology allows the consideration of other evidence of autoimmune etiology, such as replication of the disease characteristics in a tissue culture model. Our 3D tissue culture HSE model showing epithelial detachment replicates the in vivo ulceration seen in CUS, supporting an autoimmune etiology.

Autoantibodies are either of 2 types: physiological or pathogenic. Physiological antibodies are transient, they remove senescent and damaged cells as part of the normal immune response, and are not associated with a specific disease.25-27 Pathogenic autoantibodies are persistent and associated with an autoimmune disease. Properties common to pathogenic autoantibodies include (1) in vivo binding to normal tissue, (2) diagnostic specificity for 1 disease, and (3) a narrow antibody specificity, i.e., a single antigen (Table I). To prove the pathogenicity of an autoantibody, the disease’s phenotype must be induced in vivo or in tissue culture by passive transfer of the autoantibody and in animals by immunizing them with the target antigen.29 The autoantibodies in CUS do not have the common properties of physiological autoantibodies. They do, however, have the common properties of pathogenic autoantibodies. CUS patient autoantibodies bind to normal tissue in vivo, they are specific for 1 disease, and for 1 antigen ΔNp63α (Table I).

CUS autoantibodies are able to fix complement, which is a component of the innate immune system that acts to amplify the inflammatory response. The in vivo condition includes complement components unlike our experimental HSE model, which is complement free. In
the current study, an antibody concentration greater than physiological concentration was necessary to show the effects in HSEs, perhaps because of the absence of inflammatory complement components. In vivo, the complement-enhanced inflammatory reaction may exacerbate the weakened hemidesmosome attachment induced by loss of α6β4 integrin expression. An in vivo example of the effects of inflammation was illustrated in a rabbit model of autoimmune thyroiditis, where complement-deficient animals developed minimal disease.30

This study uses HSEs, a 3D in vitro tissue model, autoantibodies from CUS patients, and a monoclonal antibody to the ΔNp63α antigen to demonstrate epithelial detachment that mimics the initial stages of in vivo ulceration, leading to the clinical features of CUS. CUS autoantibodies therefore demonstrate properties of pathogenic antibodies that support an autoimmune pathogenesis. The evidence that CUS is an autoimmune disease suggests that immune-modulating drugs should be considered as potential therapeutic agents in its treatment. Our findings may be explained by the antibody-mediated abrogation of p63 function, resulting in a decrease in α6β4 integrin expression and loss of attachment of basal keratinocytes to the basement membrane. These results illustrate the critical role played by 3D tissues as a model to predict human disease pathogenesis.

REFERENCES

Table 1. Characteristics of physiological and pathogenic autoantibodies, as compared with CUS autoantibodies

<table>
<thead>
<tr>
<th>Physiological autoantibodies</th>
<th>CUS autoantibodies</th>
<th>References for CUS studies</th>
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<tbody>
<tr>
<td>Fail to bind normal tissue in vivo</td>
<td>Bind to clinically normal tissues in vivo</td>
<td>Chorzelski et al. (1998)28</td>
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<tr>
<td>Bind to altered or dead tissue antigens</td>
<td>Bind to a normal protein ΔNp63α</td>
<td>Lee et al., 19995</td>
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<tr>
<td>No diagnostic disease specificity</td>
<td>Diagnostic for CUS</td>
<td>Lee et al., 19995</td>
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<tr>
<td>Found in many or all normal sera</td>
<td>Not found in normal human sera</td>
<td>Solomon et al., 200914</td>
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<tr>
<td>Induces normal apoptosis and phagocytosis</td>
<td>Unknown</td>
<td>Causes epithelial detachment in vitro</td>
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<tr>
<td>Can play key roles in diseases</td>
<td>Causes epithelial detachment in vitro</td>
<td>Current study</td>
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CUS, chronic ulcerative stomatitis.


