Morphology and Immunohistochemistry of Spontaneous Chronic Corneal Epithelial Defects (SCCED) in Dogs

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PURPOSE. To determine the morphologic features of the epithelium and extracellular matrix in spontaneous chronic corneal epithelial defects (SCCED) in dogs.

METHODS. Forty-eight superficial keratectomy specimens were obtained after confirmation of the presence of a superficial corneal erosion for longer than 3 weeks with no discernible underlying cause. Histologic samples were examined by light microscopy, scanning electron microscopy, and transmission electron microscopy. Immunolocalization of laminin, collagen IV, fibronectin, and collagen VII was performed.

RESULTS. Epithelial cells adjacent to the defect were poorly attached to the underlying extracellular matrix. A prominent superficial stromal hyaline acellular zone composed of collagen fibrils in the area of the erosion was present in most specimens. Samples exhibited a varying degree of fibroplasia, vascularization, and leukocytic infiltrate. Laminin, collagen IV, and collagen VII were usually either not present or were present only in discontinuous segments on the surface of the erosion. Fibronectin usually coated the surface of the erosion, either as a continuous sheet or in discontinuous segments. Transmission electron microscopy of 15 samples revealed that the basement membrane was either absent in the area of the erosion or was present only in discontinuous segments. Scanning electron microscopy of eight of nine samples confirmed the absence of continuous basement membrane. Epithelial and extracellular matrix components in the peripheral cornea appeared normal.

CONCLUSIONS. Most canine patients with spontaneous chronic corneal epithelial defects do not have a normal basement membrane structure in the region of the epithelial defect and have other abnormalities in the subjacent extracellular matrix that may reflect a part of the underlying pathophysiology of chronic and recurrent erosions. (Invest Ophthalmol Vis Sci. 2001;42:2262–2269)

Spontaneous chronic corneal epithelial defects (SCCED) with no discernible underlying cause are encountered often in dogs in companion-animal veterinary practices. Affected dogs are usually middle aged, averaging 8 to 9 years of age in most studies.1–4 These erosions are characterized by sheets of loosely adherent epithelium, blepharospasm, and chronicity, with some erosions taking up to 180 days to heal.1–4 In humans, similar nonhealing erosions or recurrent epithelial erosions occur and can be associated with diverse conditions, such as traumatic abrasions, epithelial membrane dystrophy, anterior stromal dystrophies, and neurotrophic keratitis.5–8

In dogs, a variety of treatments are used with variable success rates, including simple debridement, contact lens placement, third eyelid flaps, anterior stromal puncture (ASP), chemical cautery, and superficial keratectomy (partial or complete).2–4,9–14 In addition, a variety of topical growth factors, such as epidermal growth factor, insulin, and substance P (SP), with or without insulin-like growth factor (IGF)-1, have been used.15–18 In a study evaluating techniques of treatment, superficial keratectomy was shown to have the highest success rate in healing of these epithelial defects.2 For this reason, superficial keratectomy is used by some veterinary ophthalmologists as a primary treatment.

Few ultrastructural studies have been conducted in SCCED patients with chronic erosions, despite treatment with superficial keratectomies, which provide excellent samples for analysis. One veterinary study showed an ill-defined basement membrane with basal cell abnormalities.4 The only other ultrastructural study in canine patients described a decrease in hemidesmosomes, although it was not a quantitative, controlled study.13 Several studies in dogs in which the role of SP in these erosions has been examined have shown that SP, and SP and calcitonin gene-related peptide (CGRP)-immunoreactive (IR) axons, are increased in the epithelial cells and superficial stroma of dogs with nonhealing erosions.18–20

The use of superficial keratectomy as a primary or secondary treatment for nonhealing erosions in dogs provides the opportunity to further elucidate the morphologic and extracellular matrix characteristics of this disease in dogs. Characterization and understanding of the disease in dogs may lead to a better understanding of chronic and recurrent erosions in humans.

MATERIALS AND METHODS

Superficial lamellar keratectomy was performed by board-certified veterinary ophthalmologists in 48 eyes of 46 dogs, as a therapeutic procedure, over a 6-year period (1993–1999). All animals were treated in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. All superficial, nonsectic corneal erosions had been present for a minimum of 3 weeks with no identifiable underlying cause. Before superficial keratectomy, 17 (35%) of 48 eyes had been treated unsuccessfully with epithelial debridement and ASP, and 13 (27%) eyes had been treated with epithelial debridement only. Samples were fixed in either formalin (n = 25) or 2% glutaraldehyde in phosphate buffer (n = 25). Mean age of canine patients was 8.7 ± 2.3 years (SD). Breeds represented included Labrador retriever (n = 7), boxer (n = 6), keeshond (n = 4), corgi (n = 4), mixed breed (n = 4), lhasa apso (n = 3), cocker spaniel (n = 3), poodle (n = 3),

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Boston terrier \( (n = 2) \), German shepherd \( (n = 2) \), bichon frise \( (n = 1) \), shih tzu \( (n = 1) \), dalmatian \( (n = 1) \), springer spaniel \( (n = 1) \), beagle \( (n = 2) \), chow chow \( (n = 1) \), and golden retriever \( (n = 1) \). Eleven were intact females, 12 were spayed females, 13 were intact males, and 10 were neutered males.

**Light Microscopy**

Fixed samples were routinely embedded in paraffin, sectioned at \( 6 \mu m \), and stained with hematoxylin and eosin \( (n = 48) \), Alcian blue-periodic acid Schiff \( (PAS, n = 48) \), Masson’s trichrome \( (n = 15) \), and mucicarmin \( (n = 30) \). Specific features of the epithelium that were critically evaluated included the presence or absence of a sheet of nonadherent epithelium (epithelial lip); dysmaturation of epithelium, evidenced by loss of normal order in the epithelial architecture; and the characterization and quantification of intraepithelial leukocytic infiltrate, if present. Specific stromal features evaluated included characterization and quantification of leucocytic infiltrate, if present; presence or absence of a superficial hyaline acellular zone; measurement of the hyaline acellular zone, if present; keratocyte–spindle cell proliferation; and the degree and distribution of vascularization in the sample. A mild degree of spindle cell proliferation was defined as a small number of disorganized fibroblasts, a moderate degree of spindle proliferation was defined as one to three layers of cells in a recognizable layer, and severe spindle cell proliferation was defined as more than three layers. Spindle cell proliferation was also characterized by its meridional location in the corneal sample. Quantification of leukocytes was performed by counting the number of cells in three \( \times 400 \) fields and averaging those fields. Samples were graded as having no infiltrate or a mild \( (1-10 \text{ cells/} \times 400 \text{ field}) \), moderate \( (10-20 \text{ cells/} \times 400 \text{ field}) \), or severe \( (>20 \text{ cells/} \times 400 \text{ field}) \) level of infiltrate. If a mixed population was present, the predominant cell type was recorded.

In cases in which a complete limbus-to-limbus superficial keratectomy was performed, rather than a focal excision, samples \( (n = 10) \) were also obtained from peripheral areas of cornea that appeared to be unaffected, to examine the epithelial and extracellular matrix characteristics of the unaffected cornea. These sections were processed the same as sections for light microscopy but were stained only with hematoxylin and eosin and alcian blue-PAS. The purpose of examining these peripheral specimens was to determine whether the changes observed in the region of the erosion are a feature of a more generalized disease process.

**Immunohistochemistry**

Goat polyclonal anti-collagen IV \( (n = 37, 1:80; \text{Southern Biotechnology, Birmingham, AL}) \), rabbit polyclonal anti-mouse laminin \( (n = 41, 1:40; \text{Sigma, St. Louis, MO}) \), mouse monoclonal anti-human collagen VII \( (n = 34, 1:500; \text{Sigma}) \), and rabbit polyclonal anti-human fibronectin antibodies \( (n = 37, 1:80; \text{Sigma}) \) were used in a streptavidin-biotin-peroxidase technique (Labeled Streptavidin-Biotin kit; Dako, Carpinteria, CA) after 5 minutes of digestion with proteinase K (Boehringer-Mannheim, Indianapolis, IN). Normal canine eyes were used as positive control specimens, and a negative control (primary antibody omitted) was run with each sample.
Electron Microscopy

Samples \( n = 9 \) for scanning electron microscopy (SEM) were fixed in 2% glutaraldehyde in phosphate buffer, dehydrated through an ethanol series, critical-point dried, and sputter coated with platinum after mounting. SEM was performed with a low-voltage, high-resolution scanning electron microscope (model S-900; Hitachi America, Brisbane, CA) at 1.5 keV. Samples \( n = 15 \) for transmission electron microscopy (TEM) were fixed in 2% glutaraldehyde in phosphate buffer, embedded in epoxy resin, sectioned at 1 \( \mu \)m, and examined by bright-field microscopy. Approximately 70-nm sections were then obtained from the periphery of the area of the erosion to include epithelium when possible and imaged with a transmission electron microscope (model 410; Phillips, Mahwah, NJ) with an accelerating voltage of 60 keV. In cases in which a complete limbus-to-limbus superficial keratectomy was performed \( n = 6 \), samples for TEM were also obtained in peripheral areas of cornea that appeared to be unaffected, to examine the epithelium and basement membrane of the unaffected cornea. The basement membrane was measured with image analysis software (NIH Image, provided in the public domain by the National Institutes of Health, Bethesda, MD, and available at http://www.nih.gov/od/oba) and compared with that in normal dogs by Student’s \( t \)-test.

RESULTS

Thirty erosions were located axially or paraxially, whereas 15 were located in the peripheral cornea. The location of the erosion was not noted in three samples.

Light Microscopy

Examination by routine light microscopy demonstrated 39 of 48 samples to have a sheet of epithelial cells that were not attached to the extracellular matrix adjacent to the erosion (Fig. 1). Two submitted specimens showed complete absence of an epithelial component. Forty-five of 48 samples exhibited epithelial dysmaturation characterized by loss of normal epithelial architecture. The only leukocytes found within the epithelium were neutrophils. Eighteen samples had only a mild amount of infiltrate. All other samples lacked an intraepithelial leukocytic infiltrate.

A superficial stromal hyaline acellular zone in the area of the erosion was present in 44 of 48 samples. The average measurement of the thickness of this zone was 4.405 \( \pm \) 1.563 \( \mu \)m (SD; Figs. 1A–D). The hyaline acellular zone was PAS positive, either continuously or in patchy areas in 40 of the 44 samples that had an acellular zone. Eleven of the samples with a hyaline acellular zone were stained with Masson’s trichrome, and all 11 demonstrated marked basophilic staining of the acellular zone compatible with collagen. Of the 30 samples stained with mucicarmine, 4 showed the presence of mucin on the surface of the erosion; however, 26 did not show any evidence of mucin on the surface of the exposed stroma or in the hyaline acellular zone.

Stromal leukocytic infiltrates were characterized in the area under the erosion as well as in the adjacent area (three \( \times 400 \) fields moving away from the erosion) under attached epithelium. In the stroma under the erosion, 31 of 48 samples had some type of leukocytic infiltrate. Neutrophils and lymphocytes were the predominant cell type identified (Fig. 2). In the stroma under the erosion, 17 samples had a mild, 6 had a moderate, and 3 had a severe level of neutrophilic infiltrate.

TABLE 1. Immunohistochemical Examination of Extracellular Matrix Components of Spontaneous Chronic Corneal Epithelial Defects in Dogs

<table>
<thead>
<tr>
<th>Component (n)</th>
<th>Laminin</th>
<th>Collagen IV</th>
<th>Fibronectin</th>
<th>Collagen VII</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total samples examined</td>
<td>41</td>
<td>37</td>
<td>37</td>
<td>34</td>
</tr>
<tr>
<td>Evidence of ECM component on surface of erosion</td>
<td>19</td>
<td>12</td>
<td>33</td>
<td>12</td>
</tr>
<tr>
<td>Discontinuous segments of ECM component on erosion</td>
<td>15</td>
<td>10</td>
<td>7</td>
<td>11</td>
</tr>
<tr>
<td>ECM component continuously present on surface of erosion</td>
<td>4</td>
<td>2</td>
<td>26</td>
<td>1</td>
</tr>
<tr>
<td>Normal to thickened line of ECM component under attached epithelium</td>
<td>13</td>
<td>26</td>
<td>3</td>
<td>13</td>
</tr>
<tr>
<td>ECM component attached to basal aspect of detached epithelium</td>
<td>10</td>
<td>6</td>
<td>5</td>
<td>6</td>
</tr>
</tbody>
</table>

ECM, extracellular matrix.
FIGURE 3. Immunolocalization of fibronectin (B), collagen VII (D), collagen IV (F), and laminin (H) in spontaneous canine chronic corneal epithelial defects. Note the decrease in laminin, collagen IV, and collagen VII in the area of the erosion, but the presence of fibronectin. Location of fibronectin (A), collagen VII (C), collagen IV (E), and laminin (G) in the normal canine corneal basement membrane. Staining of the basement membrane (A-G, arrows). Extension of laminin into the stroma at a presumptive ASP site (H, arrow). Magnification, ×400.
Two samples had a mild, one a moderate, and two a severe level of lymphocytic-plasmacytic infiltrate. In the stroma under attached epithelium adjacent to the erosion, 15 samples had some type of leukocytic infiltrate. Twelve specimens had a mild, two had a moderate, and none had a severe level of neutrophilic infiltrate. One sample had a mild level of lymphocytic-plasmacytic infiltrate and none had a moderate or severe level. Leukocytic infiltrate, when present, was concentrated in the superficial stroma, usually subjacent to the acellular zone. Bacteria were not observed in any of the specimens examined.

Keratocyte–spindle cell proliferation was present in 37 of 48 samples. Superficially, mild spindle cell proliferation was found in 5 samples (Fig. 1), moderate in 11, and severe in 3. In 18 specimens, generalized spindle cell proliferation was noted throughout the sampled corneal stroma, with a severe level of proliferation observed in 3 of these. This increase in the number of spindle cells appears to be a proliferative response, not an aggregation of keratocytes.

Twenty-eight of 48 specimens were vascularized. Six were vascularized only to the edge of the erosion, whereas, in 22, vascularization extended throughout the area of the erosion.

### Immunohistochemistry

Results of immunohistochemical examination of basement membrane components are shown in Table 1. Laminin, collagen IV, and collagen VII were usually either not present or present only in short, discontinuous segments on the surface of the erosion. Collagen VII, when present on the surface of the erosion, was deposited only in very thin, discontinuous, superficial strips; however, laminin and collagen IV deposition was variable, ranging from 1 to 3 μm in thickness. All three components were typically present in normal or increased amounts under attached epithelium adjacent to the erosion. Basal epithelial cells had variably positive cytoplasm as well. Laminin, collagen IV, and collagen VII were variably present as segments attached to the basal aspect of the epithelial lip. In contrast, fibronectin was commonly present on the surface of the erosion and was variably present subjacent to the epithelial lip or under attached epithelium adjacent to the erosion (Figs. 3A–H).

Results of immunohistochemical analysis of ASP samples revealed that in 4 of 15 samples, laminin extended into the stroma in the areas of stromal puncture but was absent on the surface of the erosion (Fig. 3H). Fibronectin was present on the surface of the erosion in all samples, but collagen IV and collagen VII were not identified in any ASP sites in the eight corneas examined for these extracellular matrix components.

### Electron Microscopy

Results of electron microscopy are shown in Table 2. Most samples had no basement membrane on the surface of the erosion (TEM, Fig. 4; SEM Fig. 5), basement membrane present only in discontinuous segments, or basement membrane present in a patchy distribution of abnormal basement membrane. The superficial stroma in the area of the erosion was usually composed of collagen fibrils that were sometimes (in 5/12 TEM samples) admixed with an ill-defined amorphous or
fine fibrillar material (Fig. 4B). The distribution of this amorphous, fibrillar material corresponded to the hyaline acellular zone noted on light microscopy.

**Peripheral Sections**

Six samples obtained by complete superficial keratectomy were examined by TEM in areas away from the erosion. Continuous normal-appearing basement membrane was present in all samples. The combined thickness of the basement membrane of the samples was $198.58 \pm 70.965 \text{ nm}$, which was significantly thicker than the basement membrane of normal 1- to 3-year-old dogs ($134 \pm 29 \text{ nm}; P < 0.0019$). In 10 peripheral samples examined by light microscopy, no basement membrane or anterior stromal abnormalities were detected, nor was leukocytic infiltrate present.

**DISCUSSION**

The characteristic light microscopic features of SCCED in dogs were the presence of an epithelial lip, epithelial dysmaturation, mild to moderate levels of suppurative or lymphocytic-plasmacytic infiltrate in the corneal stroma, mild to moderate spindle cell proliferation, and the presence of a PAS-positive hyaline acellular zone, which is composed of collagen. Approximately 58% of the specimens displayed some degree of vascularization. Examination of the extracellular matrix through both immunohistochemistry and electron microscopy demonstrated the basement membrane and extracellular matrix to be either absent or to be present only in discontinuous segments on the surface of the erosion.

In the normal cornea, the basement membrane remains attached to underlying stroma in superficial trauma or scrape injuries; usually, the basal epithelial cells rupture before the basement membrane attachments to the stroma are disrupted. The absence of basement membrane in SCCED dogs suggests either that adhesion complexes and/or extracellular matrix components of these patients were not normal before the occurrence of the erosion or that the normal basement membrane or its attachments do not reassemble. Normally, fibronectin, probably from the keratocytes, is thought to mediate early epithelial migration and attachment across the wound. In the specimens we examined, fibronectin was usually present, but the epithelial cells appeared not to form normal attachments, although they produced other components of the extracellular matrix and anchoring complexes (laminin, collagen IV, and collagen VII).

The normal appearance of the basement membrane in samples away from the erosion and the clinical appearance of these dogs argue against underlying primary basement membrane dystrophy. Furthermore, the consistent finding of this disease in dogs of middle to older age suggests that this healing disorder may be an age-related problem. Studies in humans show that with age, thickening of the basement membrane occurs, potentially to the extent of exceeding anchoring fibril length. Humans with diabetes may also have detachment of the epithelium with separation of the basement membrane from the underlying stroma and have been shown to have a thickened basement membrane with decreased penetration of anchoring fibrils. In specimens of epithelialized cornea peripheral to the erosion, the basement membrane of these samples was significantly thicker than that of 1- to 3-year-old dogs. However, to determine whether these samples are abnormally thickened, age-matched control subjects would have to be evaluated. In addition, these erosions are often present in varying locations throughout the cornea before surgery, and the thickening may therefore be related to the healing process. Epithelial-wounding studies in normal dogs are needed to further define the extracellular matrix changes.
Investigation of the mechanisms of basement membrane dissolution after thermal wounds has elucidated a probable role for matrix metalloproteinase (MMP)-2 and MMP-9, two gelatinases that are involved in cleaving collagen types IV, V, VII, and X and fibronectin, laminin, elastin, and gelatins. In animal models of corneal wound healing, the expression of MMP-2 and MMP-9 is increased, and the production of greater amounts of these enzymes may be associated with delayed basement membrane replacement. A recent report found upregulation of MMP-2 in the epithelium of human patients with recurrent erosions. Further quantification of MMP activity through zymography and comparisons with normal wounded and unwounded dogs would further clarify this association.

The finding of laminin in ASP–grid keratotomy sites is consistent with previous immunohistochemical studies of ASP. ASP–grid keratotomy has been reported to be successful in the treatment of this disorder in 68% to 85% of canine patients. This procedure has failed to bring about wound healing in all the patients from which our specimens were obtained; thus, we have reported only a small population of healing in all the patients from which our specimens were obtained. Of note, a superficial acellular zone has been shown to develop in rabbits after chronic epithelial scrape injury, which allows for the possibility that the acellular zone could be present merely because of the chronicity of the erosions. Of note, a superficial acellular zone has been shown to develop in rabbits after chronic epithelial scrape injury, which allows for the possibility that the acellular zone in our samples was secondary to the chronic erosion rather than the primary cause.

Modulation of the extracellular matrix by the superficial stroma would also explain why surgical interventions that impact the makeup of the stroma (ASP–grid keratotomy, superficial keratectomy, phototherapeutic keratectomy, and amniotic membrane grafts) immediately subjacent to the epithelium are often successful. It has also been shown that epithelial injury leads to apoptosis of underlying keratocytes, further emphasizing the importance of the epithelial cell-stromal interaction. These findings suggest that although the majority of studies evaluating the impact of topical therapies have been directed toward the therapies’ impact on epithelial dynamics, efforts should be directed toward the ability of these cytoactive compounds to alter the environment of the extracellular matrix.

**Acknowledgments**

The authors thank Sean Campbell for technical support.

**References**

23. Khodadoust A, Silverstein A, Kenyon K, Dowling J. Adhesion of amniotic membrane grafts40) immediately subjacent to the epithelium are often successful. It has also been shown that epithelial injury leads to apoptosis of underlying keratocytes, further emphasizing the importance of the epithelial cell-stromal interaction. These findings suggest that although the majority of studies evaluating the impact of topical therapies have been directed toward the therapies’ impact on epithelial dynamics, efforts should be directed toward the ability of these cytoactive compounds to alter the environment of the extracellular matrix.


