Pathomorphological and Immunohistochemical Evaluation of Unilateral Progressive Mooren’s Ulcer in a Cat

Mehmet Eray Alcigir1, Tuncer Kutlu2, Irem Ergin3, Sefika Karabulut4 & Gunay Alcigir5

ABSTRACT

Background: Mooren’s ulcer is a chronic and painful ulceration of the cornea. It begins progressively in the periphery and spread centrally in cornea. In human, it is seen unilaterally in most of cases. Mooren ulcer has not been reported in any kind of animals up to now. Although its aetiology is not completely enlightened, it has been suspected of the inflammatory reaction against injuries-microbiological and immun mediated effects. Immun response in presence of accumulation of immune complexes into the limbal vessels. As a result of the deficit in the regulatory mechanism because the number of suppressor cells control over B and T lymphocytes, These situations can result in a progressive tendency to inflammation because the production of autoantibodies and/or lymphokine from cytoxic T-lymphocytes creates an immune-mediated vasculitis. Numerous immigrant inflammatory cells and proteins are evaded from vessels. After triggering inflammatory cells and releasing of meditors, corneal vascularization, scar tissue and re-epithelization develop. This regenerative-reperative process plays an important role during post-inflammatory process.

Case: In this case, it was aimed to detect pathomorphological structure and immunologic relations in progressive Mooren’s ulcer (MU). A 1 year-old mix breed cat was submitted to clinic with complaints of progressive painful and eyesight loss in left eye. There were 1 cm-ulceration, opacification and old haemorrhagic areas at peripheral cornea. Histopathologically, there was wide ulceration including all layers of corneal epithelium and particularly vacuolar degeneration at suprabasal cells. In corneal stroma, numerous neutrophiles and mononuclear cells were infiltrated. Neovascularisation and fibrosis beginning from limbus were also present. This fibrotic progress was confirmed by Masson’s trichrome staining method. Immunohistochemically, Cytokeratin 3 (CK3) and cytokeratin 12 (CK12) positivities showing regenerative activity of suprabasal and basal cells were not widespread. Epidermal Growth Factor (EGF) positivities were generally weak in epithelial cells. In stroma, moderate vimentin positivities were detected proliferated in fibrocytes originating from limbus. α1-antichymotrypsin (A1AC) was mildly reacted in neutrophiles. CD3 and CD4 confirmed the presence of regulatory and helper T lymphocytes. CD3 and CD8 marked cytoxic T lymphocytes and CD20 marked B lymphocytes in inflammatory areas. CD34 were also positive in peripheral corneal stem cells derived from limbal basal epitheliums in partly regenerated area. CD57 positivity in T lymphocytes and NK cells and CD68 positivity in macrophages were attended to the area.

Discussion: CD1a positivity in T lymphocytes proved mediating lipid and microbial origin glycolipid antigens. TUNEL reactions showing DNA in situ fragmentation were present in the destructive and aging epithelial cells at periphery. In conclusion, the case has been found as unique in terms of its immunohistochemical characterization. The markers show that CD1a and CD68 expressions follow different progress in animals unlike in humanbeings even though the ulcer of pathogenetic mechanism is found identical to humanbeings.. The roles of CD20 and CD57 markers have potential roles in this ulcer. It is also concluded that insufficient epithelial regeneration, fibrosis, inflammation and apoptosis showed progressive Mooren’s ulcers having possibly microbial origin.

Keywords: Mooren’s ulcer, pathomorphology, immunohistochemistry, cat.
INTRODUCTION

Mooren’s ulcer (MU) is a chronic ulceration of the cornea. It begins peripherally and progresses centrally and circumferentially [12,17]. It is a highly rare disease when compared to other ulceration types between corneal diseases. Its etiopathogenesis has remain unknown in spite of being described by Mooren in 1867 [17]. It generally does not give response to medical treatments, and the visual outcome can be poor after surgical intervention [6,17].

Histopathologically, predominantly plasma cells and lymphocytes as well as neutrophils, eosinophils and mast cells stroma on basis of autoimmun disorder are seen in unilateral nonprogressive form. There have been necrotic debris, free erythrocytes and inflammatory cells at the peripheral part of the ulcer in bilateral progressive form. The overlying epithelium and Bowman’s membrane are absent. However, the corneal stroma is partly affected toward to central side of the ulcer when compared to periphery [12].

There are a few report regarding its molecular characterization. In these reports, c kit, STRO-1, CD45RO, VCAM, HSP, TNF as well as the markers studied in this case were evaluated [8,24]. However, CD20 has not been reported for B lymphocytes on which is known as the surface protein at beginning of the pro-B phase (specific marker: CD45R and CD117). And also CD57 is not mentioned among above cited reports. But, the marker is reported as useful for Natural Killer cell activity and adaptive immune response against viral infected cells [21].

The authors believe that the progressive-agressive MU cases can be also encountered in animals.

CASE

A 1 year-old mix breed cat was submitted to clinic of Department of Surgery, Ankara University, Faculty of Veterinary Medicine with complaints of progressive painful and eyesight loss and mucopurulent discharge for 1 1/2-month. Previously, it was learned from anamnesis that the cat was Feline Immunodeficiency virus positive after diagnosis with FIV Combo rapid test in a private clinic. In clinical examination, a corneal ulceration was detected in left eye. Conjunctiva of left palpebra inferior was also hyperemic. Topical corticosteroids (dexamethasone 0.1% eye drops) were administered for regression of inflammation during four weeks. Because corneal ulcer did not show any improvement and evaded to central area, the eye was extirpated.

The eye globe was sent to Department of Pathology for diagnosis. The material was evaluated macroscopically. It was put into 10% buffered formalin solution by being injected into inside. After fixation, transcoronal sections comprising the corneal ulcer area were done on eye globe. The tissue samples were placed into histocassettes and processed routinely. After embedding in paraffin wax, the blocks were cut at 4 micron thickness. The sections were taken onto nonadhesive slides for haematoxylin and eosin (HE) and Masson’s trichrome stainings and adhesive slides for immunohistochemistry, respectively.

Immunohistochemistry was carried out using indirect immunoperoxidase method (ABC-P). After deparaffinization and dehydration, peroxidase activity was blocked for 30 min by peroxidase blocking reagent (Novocastra Peroxidase Detection Systems, Readyto-use). Trypsinization was performed by using 0.1% trypsin solution. Thereafter antigen retrieval solution of sodium citrate (pH= 6.0) [Citrate Buffer 10x, pH=6.0, Bio Optica, BO-15-M103] was used to rest of antibodies excepting cytokeratin (CK) 3,12, vimentin, Epidermal Growth Factor (EPGF) under 750 W for 15 min keeping in microwave oven. Non-specific proteins were blocked with protein blocking reagent (Novocastra Peroxidase Detection Systems, Readyto-Use, RE7120-CE). The sections were incubated with all primary antibodies at 45°C for 1 h (Table 1) and followed by biotynlated link antibody and Horse Radish Peroxidase (HRP) antibody incubation which were applied respectively (Novocastra Peroxidase Detection Systems, Ready-to-Use, RE7120-CE). The sections were incubated with all primary antibodies at 45°C for 1 h (Table 1) and followed by biotynlated link antibody and Horse Radish Peroxidase (HRP) antibody incubation which were applied respectively (Novocastra Peroxidase Detection Systems, Ready-to-Use, RE7120-CE). The sections were incubated with all primary antibodies at 45°C for 1 h (Table 1) and followed by biotynlated link antibody and Horse Radish Peroxidase (HRP) antibody incubation which were applied respectively (Novocastra Peroxidase Detection Systems, Ready-to-Use, RE7120-CE). 3,3′-diaminobenzidine (DAB-Novocastra Peroxidase Detection System, RE7120-CE) was selected as chromogen and the sections were kept for 5 min. For counterstaining, Gill’s haematoxylin was used. The sections were mounted with nonaqueous mounting medium. For negative tissue control, the tissue sections from healthy cornea were treated with those antibodies. And also, PBS instead of primary antibodies was dropped on ulcerated cornea sections for negative reagent control. For positive tissue controls, CK3,12 was treated with rabbit skin, EPGF with neonatal mouse skin, vimentin with mouse skin, Alpha 1 Antichymotrypsin (A1AC) with rabbit tonsil, CD1a with mouse thymus, CD37-CD4-CD8-CD20-CD34-CD57 and CD68 with mouse tonsil.
The remain of procedures was the same with the others. For detection of programmed cell death by TUNEL reaction, DNA fragmentation was assessed in situ in sections using the terminal deoxynucleotidyl transferase (TdT)-mediated dUTP-digoxigenin nick endlabelling (TUNEL) method following catalog procedure (In Situ Cell Death Detection Kit, POD, Roche). The remain of procedures were the same in immunohistochemistry. For a positive control, the sections were incubated with DNase I (3000 U/mL in 50 mM Tris-HCl, pH 7.5, 1 mg/mL BSA) for 10 min at room temperature before labeling procedure. For a negative control, the samples were treated with labeling solution instead of TdT. All sections were examined using a light microscope (Euromex) and semiquantitatively score calculating after counting haphazardly labelled corneal epithelial (superficial, suprabasal, basal, limbal cells), inflammatory cells (macrophages, lymphocytes and neutrophyle leucocytes) and fibrocyte-fibroblasts on immunoreacted areas at 10 high-power fields (HPF; 400x magnification). The scoring system was graded as: negative (no staining), mild (10-30%), moderate (30-70%) and strong (70-100%).

Macroscopically, the ulcerative area was in 1 cm in diameter located at peripheral cornea. In addition, a light opacification and old-haemorrhagic areas with brownish red color were attended on the ulceration at corneal periphery. Histopathologically, structure of intact epithelial cells and basement membran integrity were destructed in a widely field. On the other hand, vacuolar degeneration was seen in especially suprabasal cells. Numerous neutrophil leucocytes, other mononuclear cells (mostly macrophages and lymphocytes) were infiltrated to stroma. Small capillaries showing neovascularisation and fibrosis predominantly composed of fibrocytes were located from limbus into

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**Table 1. Panel of primary antibodies used in the study.**

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Type</th>
<th>Dilution</th>
<th>Manufacturer information</th>
<th>Antigen Retrieval</th>
<th>Using aim</th>
</tr>
</thead>
<tbody>
<tr>
<td>cytokeratin 3-12</td>
<td>mAb mouse anti rabbit</td>
<td>1:40</td>
<td>US Biological</td>
<td>No</td>
<td>Detecting of regeneration in corneal epithelium and limbal basal cells</td>
</tr>
<tr>
<td>EPGF</td>
<td>pAb rabbit anti mouse</td>
<td>1:100</td>
<td>Sigma</td>
<td>No</td>
<td>Detecting of migrational corneal epithelial cells</td>
</tr>
<tr>
<td>Vimentin, v9 clone</td>
<td>mAb mouse anti pig</td>
<td>1:100</td>
<td>Milipore</td>
<td>No</td>
<td>Detecting of fibrocytic and fibroblastic activity</td>
</tr>
<tr>
<td>A1AC</td>
<td>pAb rabbit anti human</td>
<td>1:100</td>
<td>Abcam</td>
<td>Yes</td>
<td>Detecting of keratinocytic activity after inflammation during corneal remodeling</td>
</tr>
<tr>
<td>CD1a, Mtb1 clone</td>
<td>mAb anti mouse</td>
<td>Ready to use</td>
<td>Leica</td>
<td>Yes</td>
<td>Detecting of limbal cell proliferation</td>
</tr>
<tr>
<td>CD3, Ps1 clone</td>
<td>mAb anti mouse</td>
<td>Ready to use</td>
<td>Leica</td>
<td>Yes</td>
<td>Detecting in T lymphocyes and Langerhans cell proliferation of corneal epithelium</td>
</tr>
<tr>
<td>CD4, 4b12 clone</td>
<td>mAb anti mouse</td>
<td>1:100</td>
<td>Leica</td>
<td>Yes</td>
<td>Detecting of primarily T helper lymphocytes</td>
</tr>
<tr>
<td>CD8, 4b11 clone</td>
<td>mAb anti mouse</td>
<td>Ready to use</td>
<td>Leica</td>
<td>Yes</td>
<td>Detecting of primarily T cytotoxic lymphocytes</td>
</tr>
<tr>
<td>CD20, L26 clone</td>
<td>mAb anti mouse</td>
<td>Ready to use</td>
<td>Leica</td>
<td>Yes</td>
<td>Detecting of B lymphocytes and stromal stem cells</td>
</tr>
<tr>
<td>CD34, QBend/10 clone</td>
<td>mAb anti mouse</td>
<td>1:100</td>
<td>Leica</td>
<td>Yes</td>
<td>Detecting of B lymphocytes, stromal stem cells and epithelial cells</td>
</tr>
<tr>
<td>CD57, 514h12 clone</td>
<td>mAb anti mouse</td>
<td>Ready to use</td>
<td>Leica</td>
<td>Yes</td>
<td>Detecting of stromal NK cells and macrophages</td>
</tr>
<tr>
<td>CD68, nk1 clone</td>
<td>mAb anti mouse</td>
<td>Ready to use</td>
<td>Leica</td>
<td>Yes</td>
<td>Detecting of stromal NK cells and macrophages</td>
</tr>
</tbody>
</table>

mAb= monoclonal antibody; pAb= polyclonal antibody; RTU= Ready to use.

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corneal stroma. This fibrotic progress was confirmed by Masson’s trichrome staining method (Figure 1A and B). Immunohistochemically, vimentin was moderately expressed in proliferative fibrocyes and fibroblasts from limbus to stroma (Figure 1C). In corneal epithelium, CK3-12 mildly reacted and did not give widespread reactions. The positivities showed some regenerative activity in suprabasal and basal epithelial cells (Figure 1D). EPGF positivities were generally weakly in other epithelial cells excepting basal cells (Figure 1E). However, DNA in situ fragmentation reactions were found moderately and strongly positive in nuclei of the degenerated epithelial cells as well as stromal cells at both periphery and centrum of the cornea (Figure 1F). A1AC was moderately reacted in neutrophils (Figure 1G). CD1a was strongly positive in basal cells of limbal, suprabasal and superficial cells (at close location to limbus) peripheral and lymphocytes in stroma of peripheral cornea (Figure 1H). CD3 was moderately positive in lymphocytes and Langerhans cells (Figure 1I). Moreover, CD4 and CD8 positivities were found almost same with each other excepting positivities in central epithelial cells and stromal lymphocytes (Figures 1J and K). Additionally, CD20 and CD34 positivities were found moderately in superficial cells and strongly in B lymphocytes and possible stromal stem cells (Figures 1L and M). On the other hand, some mild positivities were obtained from CD57 and CD68. The reactions were very similar to superficial epithelial cells of corneal limbus and periphery. There were also moderate positivities in all stromal NK cells and also macrophages (Figures 1N and O) [Table 2].

**DISCUSSION**

It is hypothesized that pathogenesis of Mooren’s ulcer has been resourced by deposition of immune complexes into the limbal vessels. Suppressor cells sometimes can lose their control over B and T lymphocytes; or else, huge amount of IgA can be secreted. These situations can be resulted in the production of autoantibodies and or lymphokine from cytotoxic T-lymphocytes after blocking macrophage infiltration. After a while, an immune-mediated vasculitis is developed relating to accumulation of this products [1]. As result of this reaction, a damage at vessel’s wall is carried out. Numerous immigrant inflammatory cells and proteins are evaded from vessels [10,11]. Under these circumstances, several cytokines such as interleukin (IL)-1β, -6,-8, tumor necrosis factor-α (TNF-α) and proteases were secreted from inflammatory cells

<table>
<thead>
<tr>
<th>Localization</th>
<th>Antibody</th>
<th>Epithelium</th>
<th>Stroma</th>
<th>Bowman’s membrane</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>CK3-12</td>
<td>EPGF</td>
<td>Vimentin</td>
</tr>
<tr>
<td>Limbus</td>
<td></td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Periphery</td>
<td></td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Central</td>
<td></td>
<td>-</td>
<td>-</td>
<td>+++</td>
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<tr>
<td>Limbus</td>
<td></td>
<td>-</td>
<td>+</td>
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<tr>
<td>Periphery</td>
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<tr>
<td>Central</td>
<td></td>
<td>-</td>
<td>+</td>
<td>++</td>
</tr>
</tbody>
</table>

Table 2. Results of positivities and localization of the marker expressions and DNA in situ fragmentation in cornea.

CK3-12: cytokeratin 3-12; EPGF: Epidermal Growth Factor; A1AC: Alpha 1 Antichymotrypsin; CD: Cluster of Differentiation; Apoptosis: DNA *in situ* fragmentation Scoring: - - no staining +: mild (10-30%) immunopositivity, ++: moderate (30-70%) immunopositivity, +++: strong (70-100%) immunopositivity.
From one side, neutrophile leucocytes release collagenase, the other side the chemotactic factors lead to stimulation of progenitor cells and keratinocytes [2]. Afterthat, multipotential progenitor cells begin to differentiate into vascular endothelial cells, fibroblasts, and cornea epithelial cells. In this context, developing of corneal vascularization, scar tissue and re-epithelization make meaningfully contribution to the regeneration process after inflammation. Pathogenetic mechanism has been thought under such condition in direction of the results from this study. To the best of author’s knowledge, Mooren’s ulcer with immunohis-
In conclusion, all selected markers should detect immunological process and make a meaningful correlation with infectious agent or immunodeficiency situation in incurable MU cases.

MANUFACTURERS
1Leica Biosystems Newcastle Ltd. Nussloch, Germany.
2Bio Optica SPA. Milano, Italy.
3United States Biological. Salem, MA, USA.
4Sigma-Aldrich Corporation. St. Louis, MO, USA.
5Abcam Inc. Cambridge, MA, USA.
6Roche Diagnostics GmbH. Mannheim, Germany.

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REFERENCES


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