Corneal Neo-vascularization and VEGF Expression in Pinnipeds

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The inability of mammals of the pinniped order to induce corneal neo-vascularization has been implicated in the high rate of abnormal ocular pathology in these mammals. Captive populations of pinnipeds exhibit frequent keratitis and ocular lesions. Corneal vascularization in terrestrial mammals is triggered by protein factors released from stressed or hypoxic cells. Vascular endothelial growth factor (VEGF) is a primary protein factor involved in regulating neo-vascularization. We used immunohistochemistry and light microscopy to identify neo-vascularization in diseased pinniped corneas. We also quantified the presence of VEGF-A and VEGFR-2 protein factors in pinniped corneas with ocular insult as compared to that in normal and diseased canine corneas. Using H&E staining, we found support for the initiation of neo-vascularization in pinniped corneas; however, the extent of vascularization observed was less than that observed in diseased canine corneas. Immuno-fluorescent staining of the pinniped cornea indicated limited VEGF-A and VEGFR-2 expression. The observed high rate of abnormal pathology in pinnipeds cannot, therefore, be explained by a lack of VEGF expression. It may be that the expression of VEGF is not adequate to effectively induce vascularization to the extent seen in canines, or other factors and regulatory molecules involved in the neo-vascularization pathway, such as chaperone proteins, may explain the limited observed angiogenesis.

INTRODUCTION

Corneal angiogenesis is a standard response to corneal insult in terrestrial mammals. Aquatic mammals, however, have varied patterns of corneal vascularization. In manatees, blood vessels begin to develop in the fetus and branch extensively through normal corneas in mature animals (Harper 2005). Studies of ocular pathology in the Pinniped order – which includes walruses, eared seals (Otaruids), and ear-less seals (Phocids) – have revealed a general lack of neo-vascularization in these mammals even after corneal insult. Both free-ranging and captive pinnipeds have been found to have high rates of ocular lesions. Captive pinnipeds also exhibit frequent keratitis (Colitz et al. 2010). In captive populations, these conditions have been attributed to chemical and light damage caused by inadequate living conditions, but there are few studies confirming these ideas (Gage 2011). In attempts to find a physiological cause for the observed high rate of abnormal pathology, the lack of ability in pinnipeds to induce corneal vascularization has been identified.

Research conducted on corneal angiogenesis in terrestrial mammals has identified certain protein factors that are released by metabolically stressed or hypoxic cells and act as triggers for neovascularization. These protein factors include vascular endothelial growth factor (VEGF) and angiopoietin. A study conducted in mice by Asahara et al. (1998) revealed that the presence of VEGF alone is sufficient to induce corneal neovascularization in mice. Neither of the angiopoietin protein factors were able to cause postnatal corneal neovascularization in mice in the absence of VEGF. VEGF implants alone, however, proved sufficient to induce corneal neovascularization in not only mice but also rabbits (Coman et al. 2010) and dogs (Murata et al. 2000). VEGF has been shown to be present in the cornea and limbus of terrestrial mammals at basal levels. It is upregulated following injury to the cornea or other ocular insult that results in hypoxia (Chen et al. 2012). Inhibition of VEGF expression has been correlated with decreased vascular response following corneal insult (Stevenson et al. 2012). We looked at VEGF expression in pinniped corneas, both normal and diseased, in an attempt to determine whether null or inadequate levels of VEGF expression could explain lack of neo-vascularization in these mammals.

METHODS

We used immunohistochemistry to monitor expression of VEGF in normal and diseased pinniped corneas. We
sectioned 5 micron specimens from 40 paraffin-embedded pinniped eyes loaned from the Histopathology Lab (College of Veterinary Medicine, University of Wisconsin, Madison, WI), courtesy of Dr. Richard Dubielzig. The use of these specimens for histology was approved under IACUC protocol #201105363. Each specimen had a corresponding eye submission form, detailing any abnormality in the embedded eye. These forms were used to separate the pinniped specimens into 19 “normal” and 21 “diseased” specimens. In addition, 5 micron specimens were procured from 6 paraffin-embedded canine eyes – 1 normal and 5 diseased – from the Ocular Histology Lab (College of Veterinary Medicine, University of Florida, Gainesville, FL). Canine corneal neovascularization has been well documented (Aguirre et al. 2009). The canine specimens acted as a standard against which the vascular response and VEGF expression observed in the pinniped corneas was judged. Diseased canine eyes were also used as a positive control, to ensure the efficacy of our antigen retrieval and staining methods.

Standard immunohistochemical protocol was followed, including deparaffinization of the pinniped sections, placing the slides in xylene (2 washes, 2 minutes each), absolute ethanol (2 washes, 3 minutes each), then 90% ethanol and 80% ethanol successively (3 minutes each). Antigen retrieval was not conducted.

Each tissue section was flooded with 3% hyprogen peroxide and incubated for 20 minutes at room temperature to stop any endogenous peroxidase activity. One section from each ocular specimen was stained with hematoxylin and eosin (HE stain).

A solution of donkey serum-blocker was used to reduce background staining prior to application of our primary antibody. Then goat, polyclonal, IgG primary antibodies (ordered from the Santa Cruz Biotechnology company) were used, diluted 1:200 in PBS. These antibodies had not been previously used on pinniped tissue. They were chosen for their wide reactivity in terrestrial mammals. Two sections from each ocular specimen had primary antibody applied, either VEGF-A or VEGF-R2. In each set of slides, a pinniped section flooded with PBS instead of primary antibody was included to act as a negative control. Sections were incubated overnight at 4°C.

The following day, biotinylated secondary antibody was applied (polyclonal, donkey anti-goat IgG). This antibody was diluted 1:200, and the sections were incubated for 30 minutes at room temperature. Next, avidin-biotynylated horseradish peroxidase was applied, and the sections were incubated for 30 minutes at room temperature. All incubation periods were carried out in a humidity chamber, and multiple PBS rinses punctuated each step.

ImmPACT AEC peroxidase substrate was used to visualize VEGF expression. The AEC substrate was prepared as directed by the manufacturer. Slides were incubated in AEC chromagen until suitable stain coloration was exhibited – about 8 minutes. AEC chromagen fluoresced red in natural light.

A double-blind grading method was used to assess our results. Each section was assigned a numerical value between 0 and 3 depending on the intensity of observed staining. 0 corresponded to no observed staining; 1 to low intensity and localized staining; 2 to low intensity, widespread staining; and 3 to very intense positive staining. Four appointed “graders” were shown two images typical of each of the four staining levels. They were then given (independently) four to six micrographs representative of a slide section. They used these images to assign that section a numerical value. The number values from each grader were averaged to determine the intensity of staining on that particular slide.

RESULTS

Pinniped corneas exhibited limited expression of VEGF-A and VEGF-R2 (Fig. 1). The expression of VEGF-A was significantly lower in pinniped than diseased canine corneas (p=0.0272) (Table 1). VEGF-R2 expression was also less intense in pinniped corneas than in canine corneas; however this difference was not significant (p=0.102) (Table 1).

![A) Canine VEGF-A B) Pinniped VEGF-A](image1)

![C) Canine VEGF-R2 D) Pinniped VEGF-R2](image2)

Figure 1. Images of VEGF expression (indicated by arrows) in diseased pinniped and canine corneas (magnification: 400x).

<table>
<thead>
<tr>
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<th>Diseased Pinniped</th>
<th>Diseased Canine</th>
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<tbody>
<tr>
<td>VEGF-A</td>
<td>0.882</td>
<td>1.750</td>
</tr>
<tr>
<td>Standard Deviation</td>
<td>0.697</td>
<td>0.612</td>
</tr>
<tr>
<td>VEGF-R2</td>
<td>0.456</td>
<td>1.063</td>
</tr>
<tr>
<td>Standard Deviation</td>
<td>0.397</td>
<td>0.747</td>
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Table 1. Average numerical “grade” for diseased pinniped and canine corneas with respect to VEGF-A and VEGF-R2.


Expression of VEGF-R2 was less intense than that of VEGF in both canines (p= 0.561) and pinnipeds (p=0.062). There was no statistically significant difference in VEGF expression between normal and abnormal pinniped corneas (p= 0.684) (Table 2).

Normal canine corneas exhibited 0 VEGF expression, while abnormal canine corneas exhibited an average expression intensity of 1.406 (Table 3).

Table 2. Average Numerical Grades for VEGF Expression in Normal And Diseased Pinniped Corneas

<table>
<thead>
<tr>
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<th>Normal Pinniped</th>
<th>Diseased Pinniped</th>
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<tbody>
<tr>
<td>VEGF-A</td>
<td>0.913 ± 0.745</td>
<td>0.882 ± 0.697</td>
</tr>
<tr>
<td>VEGF-R2</td>
<td>0.674 ± 0.701</td>
<td>0.456 ± 0.397</td>
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</table>

Table 3. Average Numerical Grades for VEGF Expression in Normal And Diseased Canine Corneas

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<thead>
<tr>
<th></th>
<th>Normal Canine</th>
<th>Diseased Canine</th>
</tr>
</thead>
<tbody>
<tr>
<td>VEGF-A</td>
<td>0 ± 0</td>
<td>1.750 ± 0.612</td>
</tr>
<tr>
<td>VEGF-R2</td>
<td>0 ± 0</td>
<td>1.063 ± 0.747</td>
</tr>
</tbody>
</table>

There are two potential sources of error in this study that should be addressed. First, the use of micrographs for quantifying the level of VEGF expression in the cornea could result in a skewed numerical grade if the images are not representative of the slide as a whole. The micrographs were used to reduce variation in grades due to differences in experience (with regard to microscope use and visual accuracy) among our graders. Low intensity staining was more noticeable and more clearly defined in images from the camera than it was through the microscope lens; therefore, any bias in the results would tend to elevate the numerical score received by a specific slide. Because this grading method was used for both canine and pinniped stained sections, any basic skew in the present results should affect pinniped and canine eyes similarly, failing to affect the comparison of pinniped to canine eyes upon which the present conclusions are based.

Secondly, the lack of statistically significant difference in VEGF expression between diseased and normal pinniped eyes is interesting, especially in light of the significant difference represented in the canine corneas. This finding could be due to low levels of abnormal ocular pathology in pinniped eyes considered “normal” from a clinical standpoint. Because pinnipeds, especially those that are kept in captivity, exhibit very high levels of abrasion and disease in their cornea, it may be difficult to find a truly “normal” pinniped ocular specimen.

Nonetheless, we definitively observed corneal neovascularization in pinnipeds. In light of these findings, the observed high rate of abnormal ocular pathology among pinnipeds cannot be explained, as we hypothesized, by lack of ability to express VEGF proteins. The VEGF, neovascular pathway may still play a role in the high rate of abnormal pathology in pinniped corneas. The level of VEGF expression may be inadequate to successfully respond to ocular insult. A more likely hypothesis is that a related regulatory or chaperone protein involved in the neovascular pathway is not expressed (or is inadequately expressed) in the pinniped cornea. Angiopoietin, fibroblast growth factor, and platelet-derived growth factors are all involved in endothelial cell proliferation and neovascularization. More recently, destabilizing “tilted” or oblique proteins, have been shown by Nguyen et al. (2006) to inhibit endothelial cell proliferation and angiogenesis. Future immunohistochemical studies should focus on isolating the factor(s) involved in maintaining avascularity in the pinniped cornea, and should further document the extent of neo-vascularization observed in pinniped corneas.

Neovascularization and angiogenesis are fundamental to tumor growth and metastasis. Currently, most cancer therapies focus on inhibiting VEGF expression via created chimeric proteins (Aiello et al. 1995). However, a better understanding of the regulatory pathways which maintain avascularity in the pinniped cornea may lead to more effective treatment solutions that focus on endogenous anti-angiogenic protein factors.

**DISCUSSION**

These results suggest that limited corneal neovascularization occurs in individuals of the pinniped order. Both VEGF-A and VEGF-R2 were expressed in pinniped cornea, although not to the extent seen in canines. This contradicts the anecdotal assertion that pinnipeds cannot induce neovascularization in the cornea.

Future studies should focus on isolating the factor(s) involved in maintaining avascularity in the pinniped cornea, and should further document the extent of neo-vascularization observed in pinniped corneas. Neovascularization and angiogenesis are fundamental to tumor growth and metastasis. Currently, most cancer therapies focus on inhibiting VEGF expression via created chimeric proteins (Aiello et al. 1995). However, a better understanding of the regulatory pathways which maintain avascularity in the pinniped cornea may lead to more effective treatment solutions that focus on endogenous anti-angiogenic protein factors.
REFERENCES


