Alkali burn versus suture-induced corneal neovascularization in C57BL/6 mice: An overview of two common animal models of corneal neovascularization

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A B S T R A C T

The purpose of the present study was to quantify and compare corneal hem- and lymphangiogenesis between alkali burn and suture-induced corneal neovascularization (CNV) in two commonly used mouse strains. A retrospective analysis was performed on C57BL/6 and FVB neovascularized corneas. CNV was induced by surface caustication with NaOH or intrastromal placement of three 10.0 nylon sutures. Hemangiogenesis and lymphangiogenesis extent was calculated on whole mounted corneas by CD31 and LYVE1 immunofluorescence analysis. Blood vessel growth was similar between alkali burn and suture-induced CNV in C57BL/6 mice, and between C57BL/6 and FVB sutured strains. On the contrary, corneal lymphangiogenesis was more pronounced in the C57BL/6 sutured mice versus the alkali burn group, and in the FVB strain versus both C57BL/6 models. These results indicate that significant differences occur in lymphangiogenesis, but not hemangiogenesis, in the alkali burn and suture-induced models in C57BL/6 mice. Furthermore, lymphangiogenesis is more pronounced in the albino (FVB) strain after suture placement. We suggest that the suture model has a number of advantages and may be preferentially used to study corneal lymphangiogenesis.

Corneal neovascularization (CNV) is commonly associated with a number of ocular surface diseases. Moreover, it has been a testing ground for tumor research and, specifically, anti-neoplastic agents (Kenyon et al., 1996). While originally only corneal hemangiogenesis was studied, it soon became clear that lymphangiogenesis also occurs in the cornea. Even more interestingly, this appears to be preferentially associated with clinical events such as corneal graft transplantation (Dietrich et al., 2010). A number of animal models and strains have been proposed to study CNV (Gimbrone et al., 1974; Mutthukkaruppan and Auerbach, 1979; Fournier et al., 1981). Between these, alkali burn and suture placement are widely used due to limited technical difficulties and low cost (Bock et al., 2007; Steven et al., 2011; Ferrari et al., 2013). However, the proliferation of studies using different techniques and strains is sometimes confusing (Rohan et al., 2000; Shaked et al., 2005; Shi et al., 2011); hence, it is imperative to clarify differences and similarities between those.

For this reason we retrospectively analyzed lymphangiogenesis and hemangiogenesis and time course from corneal specimens stored in our laboratory. In order to reduce the variability all the experiments were performed by the same operator following the same protocol. Specifically, we compared corneal hem- and lymphangiogenesis: (i) in the C57BL/6 mice induced by alkali burn or suture placement, and (ii) in C57BL/6 and FVB sutured strains. The retrospective analysis was performed on suture- or alkali burn-induced neovascularized corneas (6–12 corneas/group) in 6- to 10-week old C57BL/6 and FVB mice (Charles River Laboratories, Calco, Milan, Italy). All mice were treated in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research and the Italian Ministry of Health, and all protocols were approved by the Animal Care and Use Committee of the San Raffaele Scientific Institute. The corneas had been used for different experiments but were all prepared following the same protocol, which is specified as follows.

The mouse models of (i) suture- and (ii) alkali burn-induced corneal neovascularization were used as previously described (Gimbrone et al., 1974; Ferrari et al., 2013). Briefly, (i) a 2-mm
corneal trephine was placed on the cornea and centered on the pupil and then three 10.0 nylon sutures were placed intrastromally 120° apart with knots left unburied; (ii) a single 3-mm paper disc soaked in 1 N NaOH was placed on the corneal surface for 10 s and then the eyes were washed with 15 ml saline. Few days after injury, vessels started to grow from the limbal arcade toward sutures or alkali burn. Four, 7, 10 and 14 days later mice were photographed with a slit lamp (Photoslitmap, model 40 SL-P; Zeiss, Oberkochen, Germany) (Fig. 1A) and then sacrificed and corneas collected for whole-mounts.

Immunostaining for corneal neovascularization was done according to our standard protocol (Ferrari et al., 2013). Briefly, the...
corneal stroma and epithelium were separated after ethylenediaminetetraacetic acid (EDTA; Sigma-Aldrich) treatment for 30 min at 37°C and the corneal stroma was fixed for 15 min in ice-cold acetone, rinsed in PBS and blocked for 1 h at room temperature (RT) with 2% bovine serum albumin (BSA; Sigma-Aldrich).

Tissues were incubated overnight at 4°C with anti-mouse CD31 (102501, BioLegend) and anti-mouse LYVE-1 (ab14917, AbCam, Cambridge, UK) diluted 1:200 and 1:400 in 2% BSA, respectively. The corneas were washed with PBS followed by incubation with secondary antibodies (A21209 and A21206, Invitrogen, Molecular Probes) diluted 1:500 in 2% BSA for 2 h at RT. Corneal flatmounts were prepared on glass slides using the Vectashield mounting medium (Vector, Burlingame, CA) and examined by epifluorescence microscope (model CTR5500, Leica Microsystems, Wetzlar, Germany). Adobe Photoshop was used for digital reconstruction of whole-mounted corneas by superimposition of overlapping images acquired at 5× magnification (Fig. 1B). Hemangiogenesis and lymphangiogenesis were quantitatively analyzed by calculating Neovascular Area (NA) – which measures the area of corneal vessels themselves – and Invasion Area (IA) – which measures the fraction of corneal area into which the vessels extend – as described previously (Cheng et al., 2012). Briefly, the total area of the cornea was outlined using the innermost vessel of the limbal arcade as the border; the avascular area was excluded by threshold setting while the blood/lymphatic NAs were quantified via pixel using ImageJ software. IA was measured by connecting the vessel sprouts and calculating the area enclosed between this line and the limbal arcade. NA and IA were then normalized to the total corneal area. Both NA and IA were calculated on the whole cornea for all three sutures. Data were analyzed by unpaired Student’s t-test and differences between groups were considered statistically significant with P < 0.05. All results were expressed as means ± standard error of the mean (SEM).

Hemangiogenesis in C57BL/6 mice had a similar pattern in alkali burn- and suture-induced CNV models with both IA and NA...
analysis; no significant differences were seen among groups (Fig. 1C). Similarly, the FVB sutured corneas showed no differences when compared with both C57BL/6 models 10 days after injury (Fig. 1D).

Dissimilarly from what we observed for hemangiogenesis, sutured and alkali burned corneas showed a different pattern for lymphangiogenesis. Specifically, C57BL/6 sutured mice exhibited more lymphangiogenesis (P < 0.05) as compared with C57BL/6 alkali burn group (Fig. 2A, B). Furthermore, the lymphatic vascular area was significantly increased in sutured mice as compared with both C57BL/6 CNV models at day 10 after injury (Fig 2C). In other words, our data suggest that lymphangiogenesis occurs more extensively in FVB sutured mice.

Previous work showed growth patterns of corneal angiogenesis in alkali burned and sutured mice (Shi et al., 2011). Similarly to what we observed, Shi et al. described that lymphangiogenesis developed more in sutured than in alkali burned mice. However, they reported different results with regards to the hemangiogenic response. In particular, they showed that alkali burned mice exhibited more hemangiogenesis one week after injury as compared with suture group, and that blood vessels decreased on day 14. These different results could be explained by a number of factors: (i) different animal strains were used (BALB/C instead of C57BL/6), (ii) the method used to induce alkali burn was different and (iii) vessel quantification was performed with different softwares.

In summary, we quantified and compared hem- and lymphangiogenesis following two types of injuries. We demonstrate that significant differences in lymphangiogenesis, but not hemangiogenesis follow alkali burn- and suture-induced CNV. Indeed, lymphangiogenesis was more pronounced in the suture as compared with the alkali burn model. These observations may be due to different lymphangiogenesis mechanisms occurring in the two CNV models (i.e. different cytokine pattern expression). This is confirmed by Shi et al., who showed that pro-lymphangiogenic cytokines VEGF-C and VEGFR-3 were more increased in the suture than in the alkali burn model (Shi et al., 2011). It should be noted, however, that corneal tissues obtained after alkali burn are extensively damaged and opaque. This may influence the quality of images obtained, since whole-mount technique is used. Although this should theoretically affect both hem- and lymphangiogenesis, a selective effect on the latter may be hypothesized, since lymphatic vessels lack a tunica and, hence, may be more easily damaged after alkali burn. This could contribute, at least in part, to the fact that limited lymphangiogenesis was observed in alkali burn as opposed to sutured corneas.

Regardless of the CNV model used, the mouse strain significantly influenced the angiogenic response, with more pronounced lymphangiogenesis in FVB mice. This is consistent with previous findings (Regenfuss et al., 2010) showing that lymphatic vessel growth in the FVB mice was significantly greater compared with C57BL/6 and other mouse strains, in the suture model. These and our results are in line with findings by Shaked et al., who observed that the lower angiogenic response in C57BL/6 mice is associated with lower numbers of circulating endothelial progenitors (CEPs), as compared with FVB strain (Shaked et al., 2005). Moreover, Liu and coworkers provided evidence on differences between C57BL/6 and FVB mice in skin-based angiogenesis assays (Liu et al., 2010). They found thrombospondin 1 — now reported to be an endogenous inhibitor of corneal lymphangiogenesis (Cursiefen et al., 2011) — overexpressed in C57BL/6 mice. This could explain the lower lymphangiogenic response we observed in this strain.

In conclusion, our data showed that suture or alkali burn induce similar hemangiogenesis; sutures induce more lymphangiogenesis than alkali burn in the C57BL/6 mouse. Finally, lymphangiogenesis is more pronounced in the albino (FVB) strain after suture placement. Additionally, the suture model has a number of practical advantages over the alkali model: (i) it does not induce perforations that may affect the CNV analysis; (ii) the corneal tissue is better preserved, resulting in higher quality immunofluorescence staining, and (iii), as a consequence, it requires less animal sacrifice as it is more reproducible.

References


