Substance P Modulation of Human and Murine Corneal Neovascularization

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Purpose. The purpose of this study was to investigate the role of substance P (SP) in patients affected with corneal neovascularization (CNV) and in three different Tac1-knockout (KO) murine models of CNV. Methods. SP levels in tears were measured with a multiplex bead assay. The extent of human CNV was quantitated as number of affected corneal quadrants. Murine CNV was induced in both strains by means of total disepithelization, alkali burn, and intrastromal sutures. After death, CNV (blood and lymphatic) and leukocyte infiltration were quantified by CD31, LYVE1, and CD45 immunofluorescence, respectively. Trigeminal ganglia were collected for quantitative PCR IL1β quantification. Hematoxylin-eosin corneal cross sections and whole-mounted β-3-tubulin nerve staining were used to compare anatomy and nerve density of wild-type (WT) versus Tac1-KO normal mice. Results. SP tear levels correlate positively with CNV extension in patients (r = 0.49, P = 0.03). After disepithelization, Tac1-KO corneas showed reduced blood and lymphatic vascularization (~54% and ~51% respectively) compared with the WT counterpart. CD45+ leukocytes infiltrating the cornea were reduced in Tac1-KO mice as opposed to WT in the disepithelization (P = 0.0001), alkali burn (P = 0.0258), and suture (P = 0.0149) models. Tac1-KO mice showed reduced IL1β expression in the trigeminal ganglion. Normal WT and Tac1-KO corneas did not show significant differences in transparency, thickness, and nerve density. Conclusions. Our results suggest (1) the involvement of SP in human CNV; (2) the key role of SP in promoting inflammatory CNV in three different mouse models; and (3) that absence of SP is not associated with obvious ocular surface pathology in a KO model. Keywords: neovascularization, cornea, substance P, inflammation

Proper visual function requires transparency of the cornea. Inflammation and associated corneal neovascularization (CNV) are closely related in the cornea, as it is well known that suppressing cell-mediated inflammatory response results in reduced CNV, whereas a number of proinflammatory stimuli are associated with CNV promotion. Corneal infections, autoimmune, trauma, and cicatrizing disorders can all induce CNV, which is the second cause of severe visual impairment worldwide. Current treatment options are limited and associated with significant side effects. Hence, a better understanding of mechanisms controlling CNV is needed as it could result in novel and more effective therapies.

The healthy cornea, although avascular, is the most densely innervated tissue in the body. Its neuronal network is mainly constituted of sensory fibers derived from the ophthalmic branch of the trigeminal nerve. A large percentage of these fibers (~50%/60%) contains the neurotransmitter substance P (SP). SP is a member of the tachykinin family and is produced from a precursor encoded by the pre-protachykinin A (Tac1) gene. Following nerve stimulation, SP is secreted and binds to NK (neurokinin) receptors expressed on either neuronal or non-neuronal cells. This interaction has deep implications for wound healing and inflammatory modulation. First, SP stimulates adhesion, migration, and proliferation of epithelial cells, which promote wound healing. Second, proinflammatory effects of SP are well described and play a significant role in neuroinflammation: this is a complex process characterized by nerve release of a number of proinflammatory mediators, increased vascular permeability, dilation, and angiogenesis. Proinflammatory effects mediated by SP are manifold and mainly exerted via Neurokinin 1 receptor (NK1R), which is expressed on multiple cell types including macrophages, lymphocytes, and endothelial cells.

In this study, we aimed to investigate the clinical relevance of SP in patients affected with inflammatory CNV. Further, we tested the role of SP on the neovascularization and normal anatomy of knockout (KO) animal corneas.

Materials and Methods

Patients

Tear samples were collected from 14 patients (mean age, 52 years; range, 24–80 years; 4 females and 10 males) affected by CNV. A table summarizing patient demographics is shown in Figure 1A. Slit-lamp pictures were taken after tear collection, and CNV was clinically measured dividing cornea images in...
quadrannts and giving a score from 0 (absence of vessels) to 4 (presence of vessels in four quadrants).

An informed written consent was obtained from all patients, prior to enrollment at the Cornea Unit of the San Raffaele Hospital of Milan. The study was approved by the Institutional Review Board of the San Raffaele Institute and followed the Tenets of the Declaration of Helsinki.

Tear Collection
Tears were collected by minisponge application: a single polyurethane minisponge (PeleTim; VOCO GmbH, Cuxhaven, Germany) was placed over the lids margin at the junction of the lateral and middle thirds of the lower eyelids and kept in place for 1 minute, without anesthesia, as previously described.

Avoiding the tear reflex as much as possible, the sponge was recovered, placed in the narrow end of a truncated micropipette tip adapted to a sterile 1.5-mL tube, and centrifuged at 3.5 g for 5 minutes. Tear samples were immediately stored at −80°C until further analysis. Samples were obtained before any clinical tests to avoid any interference.

Multiplex Bead Assay
Levels of SP in human tear samples were measured using the Milliplex Human Neuropeptide Magnetic Panel (HNPMAG-35K;
Millipore, Burlington, MA, USA), a multiplex bead-based assay, and performed according to the manufacturer's instructions. Dilution samples (1:3) were used in the analysis. SP concentration was measured using a Luminox 100 Bioanalyzer (Luminex Technologies, Austin, TX, USA); each sample was tested in duplicate.

Mice

Eight-week-old C57BL6/N (Charles River, Calco, Italy) and B6.Cg-Tac1tm1Bbm/J (Jackson Laboratories, Bar Harbor, ME, USA) male mice were used in all experiments. Each animal was anesthetized with intraperitoneal (IP) injection of tribromoethanol (250 mg/kg) prior to all surgical procedures. To assess corneal conditions (opacity score, discard animals with eye perforations, check the presence of the suture in the intrastromal suture experiment), in vivo corneal photographs were taken using a digital camera (EOS 50D; Canon, Tokyo, Japan) attached to a slit-lamp microscope (Photomicroscope 40 SLP; Zeiss, Oberkochen, Germany) every other day after damage until the death of the animal. Opacity was evaluated using a grading score (from 0–4; 0 = completely clear, 4 = completely opaque) described previously. Carbon dioxide inhalation and subsequent cervical dislocation were applied to euthanize the animals. All experimental protocols were approved by the Animal Care and Use Committee of the Istituto di Ricovero e cura a Carattere Scientifico San Raffaele Scientific Institute, in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

Disepithelization Model

Disepithelization (10 mice per group, two independent experiments) was performed based in part on the model previously described. Briefly, mice were anesthetized, topical anesthetic applied to their ocular surface, and the entire corneal epithelium was scraped from limbus to limbus using a blunt spatula. After wounding, eyes were treated with ophthalmic moxifloxacin 1 time/day for 1 week to minimize inflammation and keep the ocular surface moist while mice were under anesthesia. Fourteen days after disepithelization, corneas were removed for further analysis.

Alkali Burn Model

A corneal alkali burn was performed as previously described. Briefly, in the right eye of seven mice, a paper disc (3 mm diameter) soaked in 0.15 N NaOH was applied for 10 seconds under slit-lamp examination. The ocular surface was washed with 15 mL normal saline solution. To prevent infection, eyes were treated with moxifloxacin, one time per day for the first 5 days. On day 14, corneas were removed for immunostaining.

Corneal Intrastromal Suture Model

Three 10-0 nylon sutures (Alcon Laboratories, Inc., Fort Worth, TX, USA) were placed intrastromally in the right eyes of anesthetized mice (six per group, two independent experiments), 1 mm away from the limbal vessel, following a demarcation of a 2-mm trephine as previously described. On day 14, corneas were collected for immunostaining.

Immunofluorescence

At the end of any treatment, corneas were dissected. Epithelium was removed after EDTA (Sigma-Aldrich Corp., St. Louis, MO, USA) treatment (30 minutes, 37°C). Subsequently, the stroma was fixed and immunostained as previously described for the following markers: rat anti-CD31 (blood vessel marker; BioLegend, San Diego, CA, USA), goat anti-H1YVE1 (lymphatic marker; AbCam, Cambridge, UK), and rabbit anti-CD45 (leukocyte marker; R&D Systems, Minneapolis, MN, USA). Corneas were counterstained with 4’,6-diamidino-2-phenylindole (DAPI, Vector Laboratories, Inc., Burlingame, CA, USA) flat-mounted, and photographed by epifluorescence microscope (Leica CTR5500; Leica Microsystems, Wetzlar, Germany). A set of six adjacent, overlapping images were acquired and remapped into a montage, obtaining a two-dimensional (2D) reconstruction of the whole cornea. Digital pictures were analyzed using ImageJ software (http://imagej.nih.gov/ij/; provided in the public domain by the National Institutes of Health, Bethesda, MD, USA), and the neo-vascularized area of the cornea was encircled by drawing a freehand region: the CNV index was calculated as the difference between the total corneal area and the avascular area, normalized for the total corneal area.

Immune cell infiltration was quantified by counting the CD45+ positive cells per field; six peripheral fields (40X, 5-μm z-stack) per cornea were taken with a confocal microscope (Leica TCS SP5; Leica Microsystems).

To evaluate the innervation rate in different mouse strains, freshly collected corneas were processed and immunostained for the nerve marker TRJ1 (rabbit anti-β3-tubulin polyclonal antibody; Chemicon, Burlington, MA, USA), as previously described. Six peripheral fields of the peripheral subbasal nerve plexus (40X, 5-μm z-stack) per cornea were taken with confocal microscope (TCS SP5; Leica Microsystems), and the total nerve length was calculated with neuronJ.

To evaluate corneal thickness, murine eyes were frozen in OCT solution (Killed; Bio-Optica, Milano, Italy) and stored at −80°C until cross-sectioning (8 μm) and staining with hematoxylin-eosin; thickness was measured with ImageJ software (National Institutes of Health).

RT-PCR and On Ganglion

RNA was extracted from five trigeminal ganglions per group using the Paris RNA/protein isolation kit (Life Technologies, Carlsbad, CA, USA); after retro-transcription, real-time PCR was performed as previously described using the Taqman Gene Expression assay (Applied Biosystems, Foster City, CA, USA) with an IL-1β primer (IL1β, Mm01343189_m1). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH, Mm99999915_g1) transcript was used as endogenous control. Results are presented as relative expression (ΔΔCT method).

Statistics

Mann-Whitney U test and Spearman correlation coefficient were used for correlation analysis. An unpaired t-test was used to evaluate the difference in corneal thickness, nerve length, neovascularization, and immune cells infiltration between wild-type (WT) and KO murine models of CNV. Corneal opacity analysis was performed with the 2-way ANOVA method. P < 0.05 was considered statistically significant. The statistical software GraphPad Prism 5.0 (GraphPad Software, La Jolla, CA, USA) was used for all analyses. All data were expressed as mean ± SEM.

Results

SP Levels in Tears Correlate With the Extent of CNV in CNV-Affected Patients

Attributes and diagnosis of all the patients participating to this study are reported in Figure 1A. Tear SP levels have been correlated with CNV grading in each patient, showing a
positive correlation \((r = 0.49, P = 0.03)\) between the two variables (Fig. 1C). Interestingly, when dividing the patients in two groups based on the vascularization rate (1/2 mild, 3/4 severe), higher levels of SP were found in the severe vascularization subset \((P = 0.0143; \text{Fig. 1B})\); higher levels of SP have been found also in patients affected by pemphigoid compared to nonpemphigoid subjects \((P = 0.049; \text{Fig. 1D})\).

**Tac1-KO Mice CNV Is Reduced Compared With WT Mice Cornea in a Disepithelization Model**

Neo-vessel formation from the limbus to the center of the cornea was analyzed 14 days after disepithelization with CD31 and LYVE1 antibodies to stain for blood and lymphatic vessels, respectively. In both cases, WT animals developed significantly more extensive vascularization than Tac1-KO mice (Figs. 2A, 2B). Blood vessel density was 59% higher in WT \((P < 0.0001, 16 \text{ animals each group; Fig. 2C})\), whereas lymphatic vessels were 97% higher compared with KO mice \((P = 0.0015, 16 \text{ animals per group; Fig. 2D})\).

**Reduced Tac1-KO Mice Corneal Inflammation Compared With WT Mice Cornea in Three Different Ocular Surface Damage Models**

We analyzed the CD45\(^+\) cell infiltration after three different vascularization-inducing treatments: disepithelization, alkali burn, and suture. Again, in all the three conditions, Tac1-KO mice showed a reduced immune cell infiltration. In the disepithelization model (Figs. 2E, 2F), we observed a 31.5% diminution of CD45\(^+\) cells \((P = 0.0001)\), whereas in alkali burn and suture, the reduction was 25.5% \((P = 0.0258; \text{Figs. 3A, 3B})\) and 27% \((P = 0.0149; \text{Figs. 3C, 3D})\), respectively. KO mice, compared with WT, showed also a reduced ganglion’s proinflammatory cytokine IL1\(\beta\) expression after disepithelization \((P = 0.028; \text{Fig. 2G})\).
Gross Morphology and Innervation of the Normal Cornea of Tac1-KO and WT Mice Are Similar

WT and Tac1-KO mice ocular surface slit-lamp photographs (Fig. 4A) showed the same corneal transparency (opacity score ≤ 0) between the two strains at the same age (8 weeks). WT and KO corneas also showed similar thickness (Fig. 4B) on hematoxylin-eosin–stained 8-μm cross sections. Total nerve density, measured on whole mount β-3-tubulin–stained corneas (Fig. 4C), was also comparable.

**DISCUSSION**

Neovascularization and inflammation are commonly associated in the cornea. In this paper, we show that SP expression in human tears is correlated with CNV severity. This, together with our finding that SP expression is higher in ocular cicatritial pemphigoid patients (OCP), strongly support the role of SP as a key mediator in ocular surface inflammation. Indeed chronic, relentless inflammation of the ocular surface is a common finding in OCP and, interestingly, four of five patients with the highest SP expression levels presented OCP.

Our data confirm the relevance of SP as a relevant therapeutic target in patients affected with inflammatory CNV and corroborate previous reports on the proinflammatory and proangiogenic effects of SP outside of the eye.

The manifold biological effects exerted by SP include the promotion of epithelial cell proliferation and migration; hence, we wondered whether absence of SP in KO mice could impair corneal integrity. Indeed, this could represent a significant disadvantage in the clinical translation of SP-blocking treatments. Interestingly, however, KO corneas did not show any gross alteration of the corneal structure, including the epithelium, in normal conditions. In addition, corneal transparency and nerve density were not different from WT animals; these data are in line with previous findings, which suggest that neurokinin 1 receptor antagonism is not associated with obvious epithelial toxicity in mice. Although it is known that SP alone may not impact on epithelial wound healing in the cornea, this complex process may well be controlled by redundant mechanisms, to ensure its efficiency; possible additional candidates include Neurokinin 2 and 3 receptors, nerve growth factor, and others.

Although the absence of SP did not obviously impair the cornea under normal conditions, our results support a proinflammatory effect of SP in the inflamed cornea. Specifically, KO mice showed consistently reduced (~30%) leukocyte infiltration and CNV. This could be due to the direct action of SP on vascular endothelial cells, which express the SP receptor NK1 and respond to the stimulus enhancing their migration, proliferation, and permeability. SP could also promote inflammation by stimulating the secretion of specific cytokines and nitric oxide by myeloid cells or through different neurokinin receptors, such as NK2R and NK3R. Among the many cytokines regulated by SP in mammals, IL8 and MIP2 are also modulated by SP in the human cornea. Their secretion leads to the recruitment of neutrophils, which suspends the immune privilege of the cornea and stimulates endothelial cell proliferation. Our finding that SP levels are strongly associated with the extent of human corneal neovascularization could have significant implications.

First, because human CNV is an area of unmet medical need, it is associated with significant visual loss worldwide and dramatically decreases the success rate of grafting procedures, anti-SP treatments may represent a significant advancement. Of note, lymphangiogenesis was even more reduced than blood angiogenesis (97% vs. 59% reduction, respectively) in Tac1-KO mice. Interestingly, SP nerves are present in lymphatic tissues and colocalize with lymphatic vessels.
vessels in rats and humans,\textsuperscript{56,57} regulating contractility and pump flow.\textsuperscript{58} This suggests a plausible regulation of lymphatic functions, including angiogenesis, by SP. Moreover, Dietrich et al.\textsuperscript{54} suggested that inhibition of lymphangiogenesis may be even more advantageous for the prevention of graft rejection. Second, our finding that SP expression is highest in a subset of CNV patients affected with OCP suggests a role of SP in this devastating ocular disorder: although this finding deserves further investigation, SP could be released by selected leukocyte populations (e.g., macrophages) infiltrating the ocular surface in OCP.

One possible limitation in our study is the Tac1-null mice model we used, which lacks both products of the Preprota-chynkin-1 precursor protein, SP, and neurokinin A; thus, we cannot be certain that our observations are the result of the absence of the sole SP. However, we previously published\textsuperscript{59,60} that selective NK1R blockade is effective in reducing inflammatory CNV. Of note, among all the neurokinins, SP is the one with the highest affinity to NK1R,\textsuperscript{61} indicating that the biological effects we observed are largely regulated by interaction of SP with NK1R. In addition, the reduction of blood and lympho-angiogenesis with NK1R antagonist treatment (33% and 51% reduction, respectively) is similar in the Tac1-KO model. Finally, the number of infiltrating leukocytes was similarly reduced in the two studies, suggesting that inflammatory modulation may be controlled by the same mechanism.

We previously postulated the existence of a cornea-trigeminal axis, which allows inflammatory transmission from the cornea to the brain through corneal nerves.\textsuperscript{62} Specifically,

\begin{figure}[h]
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\caption{WT and Tac1-KO mice corneas show comparable morphologic traits. (A) Slit-lamp representative pictures of WT and KO mice cornea transparency. (B) Hematoxylin-eosin pictures and measurement of WT and KO central cornea cross sections. (C) Confocal images and measurement of \(\beta\)-3-tubulin-stained nerves from WT and KO mice corneas. Graphs show mean values \(\pm\) SEM; statistical analysis by unpaired Student’s \(t\) test.}
\end{figure}
Substance P Modulates Corneal Neovascularization

In summary, our findings suggest the relevance of SP in the pathophysiology of CNV, specifically in the setting of severe inflammatory disorders such as OCP. Further, they support the key role of SP as a promoter of inflammatory CNV in animal models. The multiple activities of SP, and its interaction with the myriad molecules released during inflammation, require further investigation. However, we propose that SP represent an interesting target in the treatment of corneal neovascularization in humans.

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