Research Projects in Dry Eye Syndrome
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Horst Brewitt  Hannover

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Preface

Dedicated to Prof. Behrens-Baumann on His 65th Birthday

Dry eye is one of the most clinically established eye diseases worldwide. Over the last 40 years, our knowledge of this complex disease pattern has improved substantially. The official 2007 report of the International Dry Eye WorkShop is an encyclopedic review of this disease, which comprehensively summarizes our current knowledge of the pathogenesis, clinical signs, diagnostics and therapy. Nevertheless, it is essential to remain open to new research and continue to pose questions in this field.

Therefore, over the last few years in Germany, the Ressort Trockenes Auge (linked with the Professional Association of German Ophthalmologists – BVA) has regularly supported research projects on dry eye disease and other related diseases of the ocular surface.

Prof. Behrens-Baumann, director of the Universitäts-Augenklinik Magdeburg (University of Magdeburg Eye Clinic), is an active member of this independent scientific committee and has played a large role in its activities.

Theoretical and clinical research has formed a significant part of his career and of the clinic’s activities. With this in mind, we have started to initiate and promote joint research projects with young scientists aimed at investigating the complex disease pattern of dry eye and other diseases of the ocular surface.

For this reason, I wish to dedicate this book to Prof. Behrens-Baumann on the occasion of his 65th birthday. Within these pages, well-respected German-speaking scientists working in university clinics have clearly and extensively presented their current research projects. The wide ranging themes vary from experimental basic research through to clinical practices for dry eyes. This excellent contribution makes it clear to the reader that we can look forward to exciting and stimulating research in the future.

I would like to thank all the authors for their willingness to participate in this project. In this way, you have shown your appreciation towards Prof. Behrens-Baumann.

On his 65th birthday, we wish Prof. Behrens-Baumann all the best and, above all, health and joie de vivre. May he be able to further develop his many musical and artistic interests in the future, as he has done during his professional life.
Acknowledgments

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The Dry Eye Research Award of the Ressort Trockenes Auge within the BVA is sponsored by Bausch & Lomb – Dr. Mann Pharma, who at this point I would like to thank for 10 years of generous financial support.

Last, but by no means least, I would like to thank Karger Publishers for the successful production of this ‘birthday book’.

Horst Brewitt, Hannover
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Trefoil Factor Family Peptide 3 at the Ocular Surface. A Promising Therapeutic Candidate for Patients with Dry Eye Syndrome?

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Abstract
Dry eye syndrome is a widespread disease accompanied by discomfort and potential visual impairments. Basic causes are tear film instability, hyperosmolality of the tear film, increased apoptosis as well as chronic inflammatory processes. During the last decades, our understanding of dry eye syndrome has considerably increased. However, the molecular mechanisms of the disease remain largely elusive. In this context, our group focuses on trefoil factor 3 (TFF3). Among other factors, TFF3 performs a broad variety of protective functions on surface epithelium. Its main function seems to be in enhancing wound healing by promoting a process called ‘restitution’. Studies evaluating TFF3 properties and effects at the ocular surface using in vivo as well as in vitro models have revealed a pivotal role of TFF3 in corneal wound healing. Subsequent studies in osteoarthritic cartilage seem to draw a different picture of TFF3, which still needs further elucidation. This manuscript summarizes the findings concerning TFF3 in general and its role in the cornea as well as articular cartilage – two tissues which have some things in common. It also discusses the potential of TFF3 as a candidate therapeutic agent for the treatment of, for example, ocular surface disorders.

The ocular surface is constantly exposed to the external environment, and hence epithelial defects caused by injuries, infections as well as diseases may occur. Modern living has given rise to a massive increase in the incidence of vision-threatening dry eye disease; around 10–30% of the population living in industrialized countries suffer from dry eye syndrome [1]. According to the definition of the Dry Eye Workshop Study Group in 2007 [2], dry eye disease is ‘a multifactorial disease of the tears and ocular surface that results in symptoms of discomfort, visual disturbances, and tear film instability with potential damage to the ocular surface. It is accompanied by increased osmolarity of the tear film and inflammation of the ocular surface.’ Reduced production of tear fluid or changes in the tear film composition with impaired tear film stability results in diminished moistening of the ocular surface with tear fluid.
Consequently, this leads to several aftereffects, like inflammation, adaptive immune reaction (including T cell activation), apoptosis and bacterial colonization [2]. To cope with these challenges and ensure clear vision, a rapid wound healing process is necessary at the ocular surface.

Over the past 10 years, the trefoil factor family (TFF) and in particular one of its members, trefoil factor 3 (TFF3), has edged ever closer to the spotlight – particularly since TFF3 was found to promote healing processes in the organism, especially in the mucus layer lining the gastrointestinal tract. The ocular surface is also a mucosal surface. When considered together with the protective properties of TFF3, this gives rise to the hypothesis that TFF3 is a promising therapeutic agent for ocular surface defects and especially for dry eye syndrome [3].

**TFF Peptides in General and TFF3 in Particular**

The trefoil factor family consists of 3 family members: TFF1 (formerly pS2), TFF2 (formerly hSP) and TFF3 (formerly hP1.B/hITF). They are characterized by a trefoil domain of 38–41 amino acids which contains 6 conserved cysteine residues. These 6 amino acids form 3 disulfuric bonds in a Cys1-Cys5, Cys2-Cys4, Cys3-Cys6 configuration [4]. Thus, a very rigid so-called 'trefoil structure' is formed, which offers relative protection to these peptides from proteolytic degradation. Both TFF1 and TFF3 have one trefoil domain, but form dimers by a fourth intermolecular disulfuric bond. TFF2 on the other hand has a monomeric structure, but consists of two trefoil domains.

The TFF peptides are mucus associated, and show a tissue-specific expression pattern. Until now, they have been detected in the gastrointestinal tract [5], salivary glands [6], uterus and endocervix [7] as well as the mamma [8] and as a secretory component in human milk [9]. Furthermore, TFF peptides have been found in the respiratory tract [10], colocalized with oxytocin in hypothalamic cells [11], in Vater’s ampulla [12], esophageal submucosal glands [13] as well as several other tissues [14].

At the ocular surface and the lacrimal apparatus, expression of TFF1 and TFF3 has been identified in goblet cells of the conjunctiva [15, 16] as well as in epithelial cells of the lacrimal sac and nasolacrimal sac [17]. Moreover, TFF peptides have been shown to play a role in dacryolith formation within the nasolacrimal passage. Interestingly, TFF2 is present in dacryoliths, while it is normally absent from the efferent tear duct system [18]. With regard to the ocular surface, TFF3 mRNA expression was present in healthy cornea, whereas at the protein level no TFF3 could be detected. However, under corneal pathological conditions – like Fuchs dystrophy, herpetic keratitis, keratoconus as well as pterygium – TFF3 protein was detectable [19]. Furthermore, it has been shown by in vivo studies that TFF3 protein is induced after corneal epithelial injuries in mice [20].

Although a lot of TFF3-binding proteins have been characterized, the receptor that mediates TFF3 signaling has not yet been identified [21]. It is assumed that the
receptor is localized at the basolateral side of the cell membrane in epithelial cells, and is exposed only after defects in the mucus (fig. 1) [3, 5]. Recently, the low-affinity chemokine receptor CXCR4 has been identified as a receptor for TFF2 [22]. However, TFF3 should be considered as potential ligand for that receptor as well [23].

It is known that TFF peptides offer a broad variety of protective functions [14]. They directly interact with mucins and thereby influence the rheology of the mucus in general and tear fluid in particular [24–26]. Recent ex vivo studies with tear fluid from patients with dry eye syndrome seem to support this theory. In these experiments, a positive influence of recombinant human TFF3 peptide applied into the patient's tear fluid has been observed (unpublished data). Furthermore, TFF peptides participate in the packaging and secretion of mucins. The latter demonstrates the close correlation between mucins and TFF peptides, as well as the close cooperation of TFF peptides with other proteins in general [4]. Along with their contribution in the immune response [27, 28], they are also linked to tumor progression [29, 30].

TFF3 in particular shows anti-apoptotic characteristics [31–33], promotes human airway epithelial ciliated cell differentiation [34] and has motogenic properties [5]. Several groups have observed that TFF3 enhances the migration of epithelial cells into the surrounding areas [35–38]. Moreover, TFF3 plays a key role in the process of restitution. Once the surface integrity of the epithelial layer is impaired by a defect or an injury, cells of the surrounding unaffected tissue detach from the united cell structure and migrate into the affected area. This critical early phase of the migration ensures rapid re-epithelialization [39]. Studies with murine trefoil factor 3 knock-out mice (Tff3−/−) have shown that TFF3 is essential for restitution, since these mice showed an impaired wound healing process [20, 40].

**TFF3 at the Ocular Surface**

At the ocular surface, Göke et al. [35] demonstrated the wound healing potential of recombinant TFF3 in vitro in primary rabbit corneal epithelial cells. Ensuing in vivo or combined in vivo-in vitro studies in two established corneal defect mouse models led to consistent and even more interesting results [20]. Corneal defects were induced by alkali burns (0.5 M NaOH) or by excimer laser ablation, resulting in severe localized impairment and removal of the corneal epithelium, whereas the corneal stroma and endothelium remained unaffected. To identify the endogenous expression pattern, murine Tff3 protein expression was then analyzed at different time points after the lesion. As in the healthy human cornea [19], Tff3 was absent in normal murine corneal tissue, but had already been induced in the corneal epithelium 1 h after injury. After the lesion, Tff3 expression levels in the cells close to the defect area gradually increased; even after wound closure, protein levels remained relatively high in the epithelial cells. Conversely, stromal and endothelial cells were Tff3-negative at all observed time points. Inducible expression results after injury support the protective
role of TFF3. This correlates with the findings that in Tff3–/– knockout mice corneal healing is prolonged after alkali burns as well as excimer-laser-induced injuries. Wild-type mice recover from these defects after approximately 10 h, whereas in knockout mice re-epithelialization was prolonged up to 462 h. Along with this difference in healing time, the hypothesis of Tff3 playing a pivotal role is also underlined by the finding that the recovered corneal epithelium was reduced in size and quality in 50% of the knockout mice. Only a monolayer of cells could be detected in these mice, and several areas showed detachment of the epithelium from the stroma as well as infiltration with immune cells, mainly lymphocytes. Furthermore, Tff3–/– mice lack Tff3 secreted into the tear film by conjunctival goblet cells (in addition to inducible corneal peptide), which would be provided to the apical corneal epithelium in wild-type mice.

In order to verify the in vivo results, experiments were also performed in a combined in vivo-in vitro model. First, the standardized alkali-induced corneal defects were allowed to partially heal in vivo for 6 h. Afterwards, mice were sacrificed, bulbi were enucleated, pinned down on dental wax and further cultured in a 24-well plate for an additional 124 h. The healing process was observed by measuring the remaining wounded area. Similar to in vivo results, a decelerated corneal recovery was observed in Tff3–/– mice bulbi in comparison to wild-type mice bulbi (fig. 2). The difference in recovery time was around 20 h, which is still a big difference since normal murine corneal recovery is a very rapid process.

To evaluate the biological effect of exogenously applied TFF3 upon in vivo corneal healing, ocular surface defects were induced by placing an alkali-soaked filter disk (2 mm) onto the cornea for 2 min or by excimer laser ablation following ophthalmic drop medications 3 times a day for a period of 3 days. These drops contained exogenous recombinant human TFF3 (rhTFF3) in different concentrations or bovine serum albumin (BSA) as a nonspecific protein control. Wound healing was analyzed at two

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**Fig. 1. TFF3 receptor hypothesis.** The TFF3 receptor is assumed to be localized in the basolateral side of the cell membrane of epithelial cells within the mucus layer. It is exposed to its ligands only after defects. Modified from Paulsen [3].
different time points using a grading system. The treated eyes were grouped according to the remaining impairments in the wounded area, such as defect extent and intensity, inflammatory environment observed by conjunctival hyperemia, and light reflection as a sign for identifying smoothness of the ocular surface. Three categories were considered: healed, moderately healed, and defect still present. It was obvious that rhTFF3 promoted the wound healing process in a dose-dependent manner (fig. 3). The highest concentrations (5 mg/ml rhTFF3 for alkali-induced defects and 10 mg/ml rhTFF3 for excimer laser-induced defects) accelerated the recovery process best, followed by a lower dose of rhTFF3 application, compared to BSA controls.

In order to verify these data, the experiments were repeated in the combined alkali burn in vivo-in vitro model. After alkali-induced defect induction, these bulbi were

*Fig. 2. Tff3−/− mice show decelerated corneal wound healing. Alkali-induced defects in Tff3−/− mice as well as in wild-type mice were allowed to partially heal in vivo for 6 h, followed by further in vitro cultivation for up to 124 h. Photographs were taken after 9, 25, 30, 34, 48, 53, 73, 93 and 124 h, and analysis of the wounded area was performed by measuring the remaining defect area using photo software. For better visualization, corneal defects are marked in white. Wound closure was completed after 30 h in wild-type mice, whereas in Tff3−/− knockout mice healing was finished after around 53 h (taking around 20 h longer). Modified from Paulsen et al. [20].*
allowed to recover in vitro in the presence of different concentrations of rhTFF3 peptide, BSA or no additives as a control. The healing process was again documented after several time periods. A concentration of 0.1 mg/ml rhTFF3 was found to greatly enhance corneal healing in this model compared to higher concentrations of rhTFF3, BSA or untreated control (fig. 4). Cultivating the bulbi with higher concentrations of the rhTFF3 peptide seems to have no further effect on the recovery of surface integrity compared to BSA. When comparing in vivo and in vivo-in vitro results, it seems obvious that the in vivo model needs higher doses to complete rapid wound closure. However, vital circumstances have to be taken into account as these differences between in vivo and in the combined in vivo-in vitro model might be due to blinking. Parts of the topically applied rhTFF3 might be quickly removed after blinking, so lower doses remain at the ocular surface and ensure rapid re-epithelialization instead of the applied high doses. Moreover, TFF3 results are thought to be receptor-mediated, so a dose-dependent limitation in receptor activation is possible. RhTFF3 treatment with 0.1 mg/ml revealed a quite obvious enhancement in corneal healing in the combined in vivo-in vitro model. These findings correlate with the in vitro studies which found a similar concentration to be most beneficial [35]. Additionally, and even more interestingly, exogenously applied rhTFF3 also produced beneficial effects in Tff3−/− mice. The data are in line with observations in several other animal models of gastrointestinal impairments which show positive effects after TFF3 treatment [14]. Furthermore, a recently published phase-II clinical trial lends weight to this assumption. In the course of that phase-II randomized double-blind placebo-
controlled clinical study, rhTFF3 was topically applied as oral spray for prevention of oral mucositis in chemotherapy patients with colorectal cancer. Prophylactic application of both high and low doses of TFF3 produced a reduction in occurrence and severity of oral mucositis compared to the placebo-treated group [41].

Taken together – i.e. inducible expression under pathological conditions, decelerated corneal healing in Tff3−/− mice, beneficial effects of exogenously applied TFF3 on corneal healing in wild-type and Tff3-deficiency mice in corneal defect models, and the positive influence on tear fluid from patients with dry eye syndrome – clinical trial results strengthen the hypothesis that TFF3 is a promising therapeutic candidate for treating corneal injuries, as well as ocular surface impairments caused by dry eye syndrome.

**TFF3 in Articular Cartilage**

Following the promise offered by TFF3, Rösler et al. [42] conducted studies in articular cartilage since this shares characteristics with the cornea. Both tissues are

![Fig. 4. rhTFF3 enhances corneal healing in an alkali-induced in vivo-in vitro corneal defect model. Alkali-induced defects in each group of 6 corneas were allowed to partially heal in vivo for 6 h, followed by further in vitro cultivation in the presence of rhTFF3, BSA or no additives as a control for up to an additional 72 h. Photographs were taken after 12, 24, 36, 48, 60 and 72 h, and analysis of the wounded area was carried out by measuring the remaining defect area using photo software. For better visualization, corneal defects are marked in white. Treatment with 0.1 mg/ml rhTFF3 led to highly accelerated recovery, whereas increasing concentrations of rhTFF3 had no further effect. From Paulsen et al. [20].](image-url)