ORIGINAL ARTICLE

RGTA-based matrix therapy in severe experimental corneal lesions: Safety and efficacy studies

La thérapie matricielle par administration de RGTA dans le traitement des lésions expérimentales de cornée : études de tolérance et d’efficacité

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Summary Corneal alteration potentially leading to ulceration remains a major health concern in ocular surface diseases. A treatment that would improve both the quality and speed of healing and control the inflammation would be of great interest. Regenerating agents (RGTAs) have been shown to stimulate wound healing and modulate undesired fibrosis in various in vivo systems. We investigated the effects of RGTA-OTR4120® in a rabbit corneal model in order to assess its...
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Introduction

Ocular surface alterations are observed in a large variety of diseases, such as allergic keratoconjunctivitis, scarring autoimmune syndromes, keratoconjunctivitis sicca, tear film and epithelial damages induced by long-term use of preserved eye drops, or after chemical or physical injuries. A hallmark of these diseases is the impairment of the lacrimal film, associating inflammatory cytokine release and loss of its protective properties [1–3], especially at the levels of aqueous and mucous layers that contain high amounts of growth factors [4,5], mucus and glycosaminoglycans. The ocular surface tissues, namely the epithelium, basal membrane, and stroma, are variably altered depending on the origin, etiology, severity, and duration of the aggression, which may lead to fibrotic scarring reactions.

Nowadays, ocular surface tissue injuries or impairments are usually treated with vitamins, collagenase inhibitors, anti-inflammatory agents, or tear substitutes, all of them being very often of poor or transient efficacy. However, growth factors such as EGF, NGF, IGF [6–8] and matrix proteins [9] or their fragments such as fibronectin [10] were demonstrated to be effective in treating corneal lesions in experimental animal models but have not yet come to market. Recent studies with NGF have shown promising results in humans in surgical wound healings of the cornea [11,12].

In case of HSV-1 keratitis, topical FGF-2, heparin and suramin treatment revealed a significant reduction in ulcer sizes, and complete epithelial healing was achieved earlier than in placebo-treated corneas [13]. In severe cases, cyclosporine A [14–16], autologous serum [17] or amniotic membrane (AM) [18] may be used, but their...
cost, mechanisms of action and effectiveness are still in debates. The effects of amniotic membrane transplantation (AMT) could be explained by the fact that AM secretes natural anti-inflammatory antagonists to IL-1, sTNF and VEGF-R [19,20]. Protecting agents mimicking lacrimal film properties, such as tear substitutes containing synthetic or natural polymers, polyvinyl alcohol, cellulose derivatives, or sodium hyaluronate, are frequently proposed as an adjuvant, not only for treating dry eye syndrome, but also for improving other conditions of ocular surface involvement. An agent that could combine the hydrating properties of artificial tears, mechanical protective activities toward matrix components and cells, together with stimulation of epithelial wound healing, without inducing a deleterious fibroelastic scar, would therefore be of great interest.

The regenerating agent RGTA-OTR4120® belongs to a family of polysaccharides derived from dextran by chemical substitutions with carboxymethyl and sulfate groups [21,22]. RGTA are polymers engineered to mimic heparin sulfates in order to protect and stabilize the actions of heparin-binding angiogenic growth factors, such as fibroblast growth factors FGF-1 or FGF-2, TGFbeta-1, VEGF or HARP [23,24]. In vitro, RGTA favor matrix formation by enhancing collagen [25,26] and have also a main effect on collagen I and III expression [27], on glycosaminoglycan synthesis [28], and by improving extracellular matrix remodeling. RGTA also inhibit secretion by leukocytes of elastase, plasmin, and heparinase [28,29]. In vivo, local or systemic administration of RGTA has been reported to stimulate speed and quality of wound healing in various animal species and tissue injury models [30–33], in both epithelial and connective tissues, as shown in skull defect models [34,35], or in the digestive tract [36]. Intravenously injected 3H-labeled RGTA are rapidly eliminated in a healthy animal but in a wounded animal, RGTA are only detected at the site of the injury [37]. RGTA thus remain bound to the extracellular matrix during the healing process, probably stuck to the heparin-binding sites of the matrix proteins [21,38]. RGTA therefore act as tissue-protecting and wound-healing promoting agents [22]. Several studies conducted in humans have shown some benefits in wound healing in corneal ulcer and confirmed the interest of using RGTA-OTR4120® in this pathology [39,40]. These combined properties have prompted us to study the healing and protective effects of one well-developed agent of the RGTA family, the RGTA-OTR4120®, on a severe corneal ulcer model.

Materials and methods

Animals

The animals used for all experiments were male New Zealand White (NZW) rabbits weighing about 2.5 kg and purchased from CEGAV (St Mars d’Egremn, France). They were lodged in the central animal house of Paris 5 University (agreement for animal facilities n° A75-06-02 obtained from the administration of veterinary services and the police prefecture of Paris). Before the experiments, they were quarantined and acclimatized for 1 week under standardized conditions: housing in stainless steel cages, room temperature 20 ± 1 °C, relative humidity 50 ± 5%, an alternating 12-hour light-dark cycle (8 am to 8 pm), with water and food available ad libitum (EXT C15, Dietex, St Gratien, France). They were all healthy at the time of experiments and free of clinically observable ocular abnormalities. All procedures were carried out according to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research under the supervision of a Health Authority-accredited staff member for animal care and management of the Faculty of Pharmaceutical and Biological Sciences.

In vivo RGTA toxicity assessment

RGTA OTR4120® was synthesized by OTR3 SAS (Paris, France) and was found to be more than 99% pure when tested by HPLC [38]. Before testing the potential healing properties of RGTA, we checked its ocular harmlessness and good tolerance through two separate experiments, the Draize and the tolerance tests, conducted for 7 and 28 days, respectively, on five animals each, by evaluating the Draize test criteria after instillation (Table 1) [22]. According to the routine procedure for these safety tests, animals received 50 μL containing RGTA-OTR4120® at a concentration of 100 μg/mL twice a day in the right eye, while the left eye was not treated and served as control. The dose of 100 μg/mL was chosen according to previous models of wound healing tested in other systems showing this concentration was the most effective [16–19]. Each criterion was rated to finally give a total score of ocular irritation for each animal.

Corneal alkali burn model

Twenty rabbits were first sedated with an injection at the ear marginal vein of 20 mg/kg of body weight of ketamine hydrochloride (Imalgene® 500, Merial, Saint Priest, France) and the cornea was topically anesthetized with two drops of 0.4% oxybuprocaine hydrochloride (Novartis Ophthalmics, Bulach, Switzerland). Discs of polyether sulfone, 3 mm in diameter (Sopor membranes®, Gelman Sciences, Ann Arbor, MI, USA), were dipped in a 1 N NaOH solution and applied onto the central right eye corneas for 30 seconds, immediately followed with a gentle washing with 5 mL of a 0.9% NaCl solution. Left eyes were not treated. One hour after this injury, animals were randomly divided into two groups of ten rabbits each, receiving in the injured eye a single 50 μL topical application containing either RGTA OTR4120® at 100 μg/mL, or 0.9% NaCl. For 7 days after the burn, clinical signs were scored daily using the Draize test criteria [41] (Table 1). Corneal opacities and wounded area surfaces were assessed by systematically taking pictures every day using a digital camera (Canon 550 Powershot, Japan). These digital images were evaluated separately in a masked manner. In case of discrepancy, they were assessed further. The surface of de-epithelialized areas was assessed using 1 drop of unpreserved 0.5% Fluorescein Faure® (Novartis), observed under a blue light, as well as on systematic digital pictures. The fluorescent areas were expressed as a percentage of the wounded surface, as the opaque area did not change in size throughout the 7-day study period. Animals were sacrificed on the 7th day by intravenous injection of 45 mg/kg of pentobarbital sodium (Ceva, Santé Animale, Libourne, France). The eyes were removed and fixed in a 4% formalin solution. Histological studies were
performed after paraffin embedding of the eyes (Labonord, Templemars, France) and after hematein-eosin-saffron staining of 8-mm-thick sections. Slides were observed under a light microscope (Aristoplan, Leitz, Oberkochen, Germany) coupled with the Spot-RT slicer video camera and the SPOT advance software (Diagnostic Instruments, Sterling Heights, MI). Four criteria were considered: edema, fibrosis, neovascularization, and inflammatory cells. They were scored from 1 to 4: 0: absent; 1: mild; 2: moderate; 3: high; 4: very high. Treatment received by animals was masked to the investigator conducting the histological examinations.

Statistical analyses

Statistical evaluations were performed using Statview 4.55 version software (Abacus Concepts, Berkeley, CA, USA). Statistical assessment of the in vivo studies and comparisons between the different concentrations of RGTA or control treatments were carried out using the ANOVA test and Bonferroni test.

Results

In vivo studies

First, the two different experiments aimed at determining whether tolerance of RGTA OTR4120® was good using the Draize and the tolerance tests, for 7 and 28 days, respectively, showed that this compound was very well tolerated, as no sign of ocular toxicity was observed during the follow-up periods. All the Draize criterion scores remained rated at “0”, showing the total absence of ocular irritation or toxicity. To further assess the good ocular tolerance, a 28 days iterative tolerance assay was performed and no adverse reaction was detected (data not shown).

After instigating alkali burns, this unique application of RGTA tended to reduce clinical signs and enhance re-epithelialization. Animals treated with RGTA presented a decrease in the total Draize test score (Fig. 1) within the 1st week, with a trend at all time points, reaching significance

Table 1: Draize test criteria.

<table>
<thead>
<tr>
<th>Conjunctiva</th>
<th>Iris</th>
<th>Cornea</th>
</tr>
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<tbody>
<tr>
<td>A. Redness</td>
<td>D. 1 = Folds above normal, congestion, swelling, circumcorneal injection (any one or all of there, or combination of any thereof), iris still reacting to light 2 = No reaction to light, hemorrhage, gross destruction (any one or all of these)</td>
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<tr>
<td>1 = Vessels definitely injected above normal 2 = More diffuse, deeper crimson red, individual vessels not easily discernable 3 = Diffuse, beefy red</td>
<td>1 = Easily discernible translucent areas—details of iris slightly obscured 3 = Opalescent areas, no details of iris visible, size of pupil barely discernable 4 = Opaque—iris not visible</td>
<td></td>
</tr>
<tr>
<td>B. Chemosis</td>
<td>E. Degree of opacity</td>
<td></td>
</tr>
<tr>
<td>1 = Any swelling above normal (includes nictitating membrane) 2 = Obvious swelling with partial eversion of the lids 3 = Swelling with lids about half closed 4 = Swelling with lids half to completely closed</td>
<td>1 = One quarter (or less) but not zero 2 = Greater than one quarter, but less than one half 3 = Greater than one half, but less than three quarters 4 = Greater than three quarters, up to whole area</td>
<td></td>
</tr>
<tr>
<td>C. Discharge</td>
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<tr>
<td>1 = Any amount different from normal 2 = Discharge with moistening of lids and hairs adjacent to the lids 3 = Discharge with moistening of lids and considerable area around the eye</td>
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Scores were calculated as \([A + B + C] \times 2 + (D \times 5) + (E \times F \times 5)\).

Figure 1. Means (M) and standard deviations (SD) of ocular irritation scores of alkali-burned eyes based on the Draize test criteria.
at D2, D4, and D7 when compared to animals receiving saline ($P=0.04$; $P=0.02$, and $P=0.02$, respectively). The intensity of corneal opacity, consistently reproducing the area of alkali burn, with a totally opaque lesion at D1, reduced slowly in the two groups but remained present at D7, with no significant difference between the two treatments (data not shown). The fluorescein test (Fig. 2) revealed a maximal de-epithelialization at D1 (100% of opacity areas) for all animals and a rapid re-epithelialization occurring in the first few days, with a trend favoring RGTA at all time points, reaching statistical significance at D2 ($P \leq 0.05$, compared to saline-treated eyes). Histological analysis showed edema, fibrosis, new blood vessels, and inflammatory cells in all corneas, excepted for edema in the RGTA-treated group, in which this sign was observed in only four of ten eyes, mostly rated at a mild level. The scores of these four criteria were significantly lower in the RGTA-treated group than in the control group ($P=0.002$, 0.003, 0.005, and 0.002, respectively; Fig. 3). At a lower magnification, the view of the whole cornea showed no alteration of the RGTA-treated corneas. Cornea resumed its normal thickness 315.8 ± 20 microns through the whole length and no enlargement at the site of the original injury could be detected. Larger magnification confirmed this observation showing lamellar organization of the stroma with remaining microvessels. Epithelium and basal membrane were almost as mature as in a non-injured cornea. Moreover, the Descemet membrane and the underlying endothelium seemed also untouched.

In contrast, burned corneas treated with NaCl showed inflammation in the whole tissue both in length and thickness. Increases in thickness ranged from 685 ± 40 microns in the central zone of the burned cornea. Stroma was highly disorganized, especially in the anterior part where the burn occurred, but an inflammatory thickening at the level of the Descemet membrane and endothelium was also visible. Many microvessels remained present in the anterior part of the stroma. Epithelium formation was less mature, thinner than in RGTA-treated corneas.

**Discussion**

This series of in vivo experiments investigated the effects on the ocular surface of RGTA OTR4120®, a healing agent that mimics heparan sulfate action. First, we studied the in vivo action of RGTA-OTR4120® on normal rabbit eyes to confirm the absence of ocular irritation or toxicity, and then on a rabbit alkali burn model. The absence of ocular toxicity, the mild but significant enhancement of re-epithelialization, with a significant reduction in clinical signs of ocular inflammation, the major changes observed histologically in the wounded zone compared to eyes receiving saline instead of RGTA, make this compound a promising agent for improving ocular surface wound healing and matrix remodeling. RGTA could therefore play a potential role for controlling and regulating the wound healing process in various ocular surface diseases, such as those involving corneal epitheliopathy, chemical or physical trauma, severe dry eye syndrome, scarring conjunctivitis, or after refractive surgery.

Corneal ulcers and erosions may therefore resist classical treatments, especially when associated with a chronic process that induces an inflammatory reaction. In order to preserve corneal integrity and to prevent or treat corneal epithelial defects, numerous molecules have been tested, such as collagenase inhibitors, ascorbate, citrate, fibronectin, heparin, suramin, EGF, IGF or NGF, but most of them have not proven a true and complete effect and none of them if marketed are easily available. Indeed, the RGTA family presents many of the functional characteristics of these molecules: mimetic of heparan sulfates, RGTA offer similar properties similar to those of heparin [23,28], acting by binding growth factors and thus protecting them from enzymatic degradation and improving their local actions [23]. RGTA are also able to inhibit cathepsin-G [42], collagenase, and have shown anti-inflammatory effects [29,30,42,43] and wound healing-promoting activities on many tissues. They have been reported to favor tissue repair in burned skin [32,33], muscles [4,44], bone [45], after gastric ulceration [36], colon and even cornea [46], in which another heparin sulfate analog was shown to enhance re-epithelialization after corneal ulcer.

All these properties may therefore justify the development of RGTA for treating a wide range of ocular surface damages. This series of preliminary experiments aimed to determine the toxicological and functional profiles of RGTA OTR4120®.
Figure 4. Microphotographs of RGTA-treated (A) and saline-treated (B) rabbit corneas with internal scale after hematein-eosin-saffron staining of 8-mm-thick sections. (Magnification: × 40, × 160, × 400).

By conducting a Draize test with a b.i.d. application for 7 days and a tolerance test for 28 days, we first confirmed the absence of ocular irritation induced by RGTA-OTR4120® tested at the concentration considered to be the most appropriate for enhancing wound healing according to the above-mentioned models. These tests proved that RGTA-OTR4120® was not toxic and was well tolerated according to the standardized Draize criteria, even though this standard of ocular toxicity is probably not powerful enough to fully assess the absence of toxic side effects of ophthalmic drugs and needs complementary studies. We thus showed that in vivo on this rabbit alkali burn model, only a single application of RGTA-OTR4120® could improve healing, especially during the first few days, as was shown using the fluorescein test, scoring of clinical ocular signs, and histologically. Histological patterns observed after 7 days therefore showed much better wound healing patterns, with significantly less stromal edema, fibrosis, neovascularization, and inflammatory cells, in rabbits treated with RGTA-OTR4120® compared to eyes receiving saline as control. Epithelial recovery seemed to appear earlier with a unique application of RGTA-OTR4120®, but the difference was limited, because rabbit cornea spontaneously heals rapidly and possibly because a single application was too low to promote more striking wound healing effects in such a severe model. Nevertheless, reductions of inflammatory clinical signs, such as tearing, chemosis or hyperemia, as well as the improvement of histological patterns, make this single dose already a promising indication for clinical applications. This study confirms conclusions of a pilot non-controlled exploration with RGTA-OTR4120® treatment on compassion use for corneal ulcers and severe chronic dystrophies resistant to the usual treatments initiated in 2008 [39]. It reduced pain and favored corneal healing in almost all corneal ulcers. Weekly instillation of a single drop however seemed insufficient and these very promising data
need to be confirmed on a larger population in a controlled trial with more adapted dosages. Another study, a case report in 2011 described a RGTA-OTR412O® [40] treatment of herpetic zoster ophthalmicus, which leads to improvement of ulcer observed 1 week later and complete healing in less than 3 weeks, with no side effects.

In conclusion, RGTA did not induce any toxicity in vivo even at high doses. It was able to improve corneal wound healing, especially at the histological level, even after a major injury such as an alkali burn, and with a single application. The RGTA-OTR412O® molecule is stable and can be synthesized with high definition and reproducibility [38]. More than 70 preclinical studies have been published [22] on this technology, notably on skin healing [27,32,33,47] and on mobilization of stem cells [48,49], and several pilot uncontrolled clinical studies have been carried on [39,40]. As a result, RGTA-OTR412O® product is now developed as a CE marked medical device named CACICOL20® and dedicated to corneal ulcer. Today, CACICOL20® is not yet commercialized. In a previously quoted pilot clinical compassionate study performed in very severe corneal dystrophies and ulcers, CACICOL20® displayed a very good tolerance and a significant reduction in pain. These results supported the interest of conducting a controlled study and have justified the implementation of a double-blind controlled study on the healing of chronic corneal ulcers. This heparan mimetic, which may stimulate extracellular matrix healing, may be thus a possible alternative therapy to autologous serum or amniotic membrane transplantation in severe corneal ulcers and chronic keratitis resistant to current therapies. Randomized studies will have to be performed to validate these first clinical and experimental observations.

Disclosure of interest

The authors declare that they have no conflict of interest concerning this article.

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