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Effects of subconjunctival ranibizumab in a presensitized rat model of corneal graft☆

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ABSTRACT

We evaluated whether corneal graft survival in presensitized corneal transplantation was affected by subconjunctival ranibizumab in a rat model. The effect of ranibizumab in the presensitized corneal transplantation has not been previously reported, although anti-VEGF was attempted on a non-presensitized model in other studies. Corneas were transplanted from Brown Norway to Spraque Dawley rats. The recipient rats were randomly assigned to three groups: Group 1, skin autograft and subconjunctival injection of PBS; Group 2, skin allograft and injection of PBS; and Group 3, skin allograft and injection of ranibizumab (vascular endothelial growth factor antibody). A skin graft was performed 2 weeks before corneal transplantation. On days 3, 7, 11, and 14 after transplantation, the grafts were scored. The number of corneas with graft rejection on day 14 was significantly higher in Group 2 than in Group 1 or 3 (6/15 [40.0%] in Group 1, 13/15 [86.7%] in Group 2, and 4/15 [26.7%] in Group 3). The mean clinical scores for edema, opacity, and new vessels in Group 3 were significantly lower than those in Group 2, while the edema score in Group 1 was significantly lower than that in Group 2 on day 14. Before corneal allotransplantation, presensitization by skin grafting accelerated the graft rejection process. In a short-term presensitized rat model of keratoplasty, application of subconjunctival ranibizumab prevented graft rejection.

1. Introduction

Corneal allotransplantation has been widely used in clinics to treat corneal disease. A normal, healthy cornea is devoid of both blood and lymphatic vessels, but inflammatory conditions such as chemical burns or herpes infection can lead to breakdown of this "angiogenic privilege" (Cursiefen et al., 2004c). Allograft rejection is a leading cause of corneal graft failure and thus a leading indication for repeat penetrating keratoplasty (Coster and Williams, 2005). There is increasing awareness amongst clinicians that a strong risk factor for corneal graft immune rejection is the presence of pre-existing or growing blood vessels (Bachmann et al., 2010; Maguire et al., 1994; Symes and Poole, 2010; Williams et al., 1992). Regenfuss et al. showed that topical application of vascular endothelial growth factor (VEGF) antagonists, such as pegaptanib or ranibizumab, inhibited corneal neovascularization (Regenfuss et al., 2009). Bevacizumab, ranibizumab, pegaptanib, and trastuzumab were found to inhibit corneal neovascularization in a rat model (Sener et al., 2011). The reduction of pre-existing or growing neovessels using VEGF antibodies (anti-VEGF) has been shown to improve the success of high-risk allogeneic corneal transplantation in animal models (Bachmann et al., 2008; Cursiefen et al., 2004a; Papathanassiou et al., 2008; Rocher et al., 2011). However, no definite conclusions about immune rejection can be drawn from animal studies because the large amount of proinflammatory cytokines [tumor necrosis factor (TNF)-α and interleukin (IL)-1] and growth factors [transforming growth factor (TGF)-α, TGF-β, basic fibroblast growth factor (b-FGF), and platelet-derived growth factor (PDGF)] might cause graft failure after keratoplasty.

Therefore, Using a rat model, we evaluated whether corneal graft survival in presensitized corneal transplantation is affected by subconjunctival ranibizumab (anti-VEGF). To the best of our

Abbreviations: BN, Brown Norway; FGF, fibroblast growth factor; IL, interleukin; MMPs, matrix metalloproteinases; PDGF, platelet-derived growth factor; SD, Spraque Dawley; TGF, transforming growth factor; TNF, tumor necrosis factor; VEGF, vascular endothelial growth factor.

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knowledge, the effect of ranibizumab on corneal graft survival in the presensitized corneal transplantation model has not been previously reported, although anti-VEGF was attempted on a non-presensitized model in other studies. This study showed that allosensitization was important in the rat model of corneal transplantation. Skin graft before keratoplasty made allosensitization and graft rejection more quickly and effectively. Also, this study provided information about the immune reaction related to angiogenesis in grafted corneas. Graft rejection of the cornea may be related to the immune reaction, which was inhibited by anti-angiogenesis. Therefore, our results show that the inhibition of angiogenesis by ranibizumab prevented graft rejection in a short-term presensitized rat model of keratoplasty.

2. Material and methods

2.1. Animals and rat cornea transplantation

Eight-week-old Brown Norway (BN) female rats and Spraque Dawley (SD) female rats were obtained from Charles River Laboratories (Yokohama, Japan). The SD rats were anesthetized with a mixture of 125 mg/kg ketamine chlorhydrate (UVA, Ivry-sur-Seine, France) and 5 mg/kg chlorpromazine (Specia Rhône Poulenc, Paris, France). Each animal was systematically weighed before examination and/or the experimental procedures. For corneal transplantation, penetrating keratoplasty was performed by one corneal surgeon (KJC), as described previously (Bourges et al., 2006). In brief, corneal buttons from the sacrificed BN rats were obtained using a 3.0-mm trephine and were grafted into a 2.5-mm corneal bed in the left eyes of the SD rats. The day of surgery was day 0. Paracentesis was performed before trephination under maximum pupil dilation (Midrin-P; Santen Pharmaceutical Co., Osaka, Japan) and the anterior chamber was filled with viscoelastic fluid (Healon; Abott Medical Optics, IL, USA). A 3.0-mm trephination was performed using a biopsy punch and was completed with Vanas scissors. The BN rat corneal button was secured in place by eight interrupted 10-0 nylon sutures (Ethicon, Saint Stevens-Woluwe, Belgium). Ofloxacin ointment was applied at the end of surgery. No corticosteroids were used. The corneal sutures were removed after 4 days. Transplanted eyes with intraoperative or immediate postsurgical complications before day 2 (suture rupture or endophthalmitis) were excluded and replaced by the next grafted animal on the randomization schedule. All animal procedures were approved by the ethics committee of the Catholic University of Korea and were carried out in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH Publications No.80-23).

2.2. Skin transplantation: presensitization

The rats (BN and SD) were anesthetized as described above and laid out on a sterile surface, ventral side up. The tail was cleaned as far as possible by swabbing with 10% povidone iodine and the graft bed was prepared by removing a thin sliver of skin about 10 mm long, and about half the width of the tail (Fig. 1A). In the skin allograft group, the piece of skin that was removed from the BN rats was grafted to the SD rats. In the skin autograft group, the piece of skin that was removed from the SD rats was grafted to the same rats. The skin was secured in place by four interrupted 8-0 Vicryl sutures (Ethicon) (Fig. 1B). Skin transplantation was done 2 weeks before corneal transplantation. Incompatible grafts would usually be rejected within about 10–12 days, with drying and falling off (Fig. 1C).

2.3. Treatment protocol

The SD rats were randomly assigned to three different treatment groups: Group 1, skin autograft, keratoplasty, and subconjunctival injection of PBS ($n = 15$); Group 2, skin allograft, keratoplasty, and subconjunctival injection of PBS ($n = 15$); and Group 3, skin allograft, keratoplasty, and subconjunctival injection of ranibizumab (Lucentis; Genentech Inc., South San Francisco, CA, USA) ($n = 15$). Those SD rats that did not show skin graft rejection were replaced with others. The grafted rats were sacrificed on day 14 using an overdose of intraperitoneal pentobarbital (Ceva Santé Animal, Libourne, France).

2.4. Subconjunctival injection

Prior to the subconjunctival injections, the rats were anesthetized. The subconjunctival injections were performed on the day of the surgical procedure and on days 3, 6, 9, and 12. Subconjunctival injections (2 μl) were performed in grafted eyes using a 30-gauge needle (Insulin Syringe; Sungshim Medical Co., Bucheon, Korea). All injections were performed under an optical microscope and were situated at least 2 mm away from the needle puncture. To reduce leakage, conjunctival punctures were pinched with a microsurgical forceps 3 s after injection.

2.5. Evaluation of the rejection process

A biomicroscopic examination and imaging were performed on days 3, 7, 11, and 14 after surgery. When taking photographs, the pupils were dilated with mydriatics (Midrin-P) and used red reflex light from the retina to see new vessels more clearly. The progression of edema and transparency, and the growth of

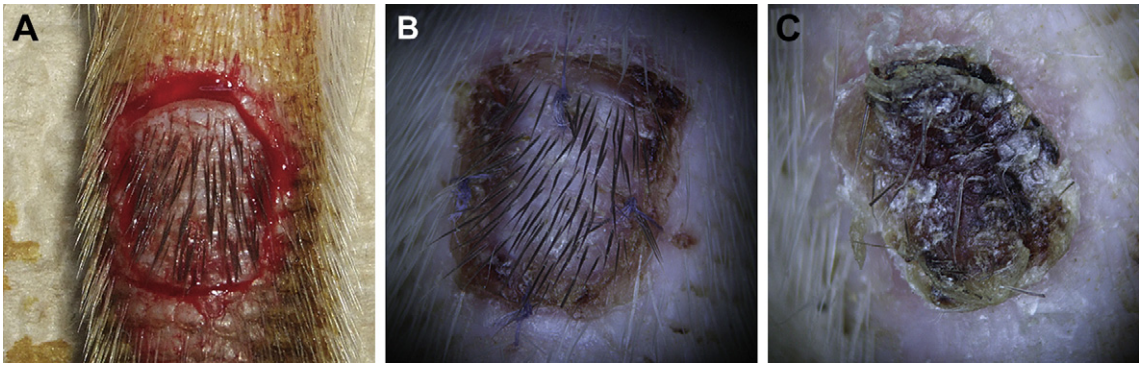

Fig. 1. Skin transplantation for presensitization before corneal transplantation. In the skin allograft group, pieces of skin that were removed from BN rats were grafted to SD rats (A). The skin was secured in place by four interrupted sutures (B). The rejected grafts dried up and fell off (C).