Role of Stromal Cell-Derived Factor-1 and Chemokine Receptor 4 in Rat Corneal Transplant Rejection: Postprint

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Abstract

Objective To investigate the expression of stromal cell-derived factor-1 (SDF-1) and CXCR4 in rat corneal tissue and their role in immune rejection following corneal transplantation.

Methods Fifteen Wistar rats served as the normal control group; 22 Wistar rats underwent autologous corneal transplantation as the autograft group; 22 SD rats and 44 Wistar rats were utilized, with SD rats as donors and Wistar rats as recipients for penetrating keratoplasty. Postoperatively, 22 rats were randomly assigned to the TobraDex group, receiving TobraDex eye drops in the operated eye (twice daily), while the remaining 22 rats were assigned to the allograft group, receiving an equivalent volume of normal saline in the operated eye for one month. Clinical evaluation of corneal grafts in each group was performed according to Larkin' s method; corneal grafts from the operated eyes were harvested on postoperative days 5 and 9 for histopathological observation, immunohistochemical examination, and real-time quantitative PCR detection.

Results No rejection occurred in the autograft group. The corneal survival time in the TobraDex group was 24±0.32 days, which was significantly longer than that in the allograft group (10±0.36 days) ($P<0.001$). Histopathological examination revealed extensive inflammatory cell infiltration and neovascularization in the corneas of the allograft group. The expression levels of SDF-1 and CXCR4 mRNA were significantly elevated in corneal tissues of the allograft group ($P<0.001$ on day 5, $P<0.01$ on day 9) and were significantly reduced in the TobraDex group compared with the allograft group. Immunohistochemical examination demonstrated that SDF-1/CXCR4 were predominantly expressed in the epithelial and stromal layers of the corneal grafts, with significantly increased content of SDF-1 and CXCR4 in corneal tissues of the allograft group.

Conclusion SDF-1/CXCR4 may be involved in early postoperative rejection

following rat corneal transplantation, potentially through a mechanism whereby SDF-1 specifically induces maturation and chemotaxis of CXCR4-positive cells toward the rejection site and promotes corneal neovascularization.

Full Text

Role of Stromal Cell-Derived Factor-1 and CXC Chemokine Receptor 4 in Corneal Graft Rejection in Rats

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Abstract

Objective: To examine the expression of stromal cell-derived factor-1 (SDF-1) and CXC chemokine receptor 4 (CXCR4) in rat corneal tissue and their role in corneal allograft rejection.

Methods: Fifteen Wistar rats served as normal controls. Twenty-two Wistar rats underwent autologous corneal transplantation (autograft group), while 44 Wistar rats received allogeneic corneal grafts from SD donors via penetrating keratoplasty. Among the allograft recipients, 22 were randomly selected for treatment with TobraDex eye drops twice daily for 30 days postoperatively (TobraDex group), and the remaining 22 received equivalent volumes of normal saline (allograft group). Clinical assessment of corneal grafts was performed using Larkin' s method. Histopathological observation, immunohistochemistry, and real-time quantitative PCR of corneal grafts were conducted on days 5 and 9 after transplantation.

Results: No graft rejection occurred in the autograft group. The TobraDex group exhibited significantly longer graft survival compared to the saline-treated allograft group (24 ± 0.32 vs. 10 ± 0.36 days, $P < 0.001$). Histopathology revealed numerous inflammatory cells and neovascularization in the allograft group. SDF-1 and CXCR4 mRNA expression increased significantly in corneal tissues of the allograft group ($P < 0.001$ on day 5 and $P < 0.01$ on day 9), while TobraDex treatment markedly reduced their expression. Immunohistochemistry showed SDF-1 and CXCR4 expression primarily in the corneal epithelium and stroma, with significantly elevated levels in the allograft group.

Conclusion: SDF-1/CXCR4 may participate in early corneal graft rejection in rats, possibly through SDF-1-mediated induction of CXCR4+ cell maturation and chemotaxis toward the graft, promoting corneal neovascularization.

Key words: stromal cell-derived factor-1; CXC chemokine receptor 4; graft rejection; corneal transplantation

Introduction

Chemokines are currently recognized as the primary factors guiding inflammatory cells to rejection sites and play crucial roles in post-transplant immune responses [1-2]. Stromal cell-derived factor-1 (SDF-1), a member of the chemokine family, mediates immune cell activation and migration through binding to its specific receptor CXC chemokine receptor 4 (CXCR4) [3-5]. While SDF-1/CXCR4 involvement in transplant rejection has been demonstrated in kidney, pancreas, and artery transplantation [6-8], no studies have investigated this pathway in corneal transplantation. Corneal transplant rejection is a complex immune response involving multiple immune cells and molecules. Despite corneal immune privilege and anterior chamber-associated immune deviation, inflammatory cells can still infiltrate corneal grafts, and rejection remains the primary limiting factor for corneal transplant failure [9-11]. To investigate whether SDF-1/CXCR4 participates in post-transplant rejection and whether inflammatory cell infiltration correlates with SDF-1/CXCR4 expression, we designed this study to explore potential new therapeutic targets for anti-rejection therapy.

1.1 Experimental Animals and Materials

Twenty-two SD rats served as donors and 66 Wistar rats as recipients for penetrating keratoplasty, with an additional 15 Wistar rats as normal controls. All rats were purchased from the Laboratory Animal Center of Southern Medical University, were female, 6-8 weeks old, weighed 180-220 g, and were specific-pathogen-free (SPF) grade. Animal use complied with laboratory animal management regulations. Anesthesia was induced with 3% pentobarbital sodium (1.5 mL/kg), and mydriasis was achieved with compound tropicamide eye drops. Ten-to-nylon sutures were purchased from Ethicon (USA), TobraDex from Alcon (USA), immunohistochemistry antibodies from Abcam (UK), RNA extraction kits, reverse transcription kits, and real-time PCR kits from Takara (Japan), and primers were synthesized by Invitrogen (Shanghai, China).

1.2 Grouping and Surgical Methods

A completely randomized design was employed. Fifteen Wistar rats without corneal transplantation served as normal controls. Twenty-two Wistar rats underwent autologous corneal transplantation (autograft group). Twenty-two SD rats served as donors and 44 Wistar rats as recipients for allogeneic corneal transplantation. On postoperative day 2, 22 Wistar rats were randomly selected for TobraDex eye drops twice daily for 30 days (TobraDex group), while the remaining 22 received equivalent volumes of normal saline (allograft group).

Donor and recipient rats received compound tropicamide eye drops for mydriasis 20 minutes before surgery and intraperitoneal injection of 3% pentobarbital sodium (1.5 mL/kg) for anesthesia. Surgery was performed under a microscope (Leica, Germany). Donor corneal grafts were obtained using a 3.5 mm trephine, and recipient beds were prepared with a 3.0 mm trephine. Grafts were sutured with eight interrupted 10-0 nylon sutures. Viscoelastic agent (Bausch Lomb, China) was injected during and after surgery to maintain anterior chamber depth. TobraDex ointment (Santen, Japan) was applied to the conjunctival sac at the end of surgery. Corneal sutures were not removed postoperatively.

1.3 Observation and Criteria for Rejection

Ten rats from each group were randomly selected for clinical rejection evaluation. Grafts were examined daily under a surgical microscope for 30 days postoperatively. Corneal parameters were recorded according to Larkin' s scoring method [12]. Rejection was defined as a total score 5 or a transparency score of 3. Rats with severe infection, anterior chamber hemorrhage, lens opacity, extensive iris adhesion, wound dehiscence, or lens prolapse within 3 days post-surgery were excluded and replaced.

1.4 Histopathology and Immunohistochemistry

On days 5 and 9 postoperatively, three rats from each group were randomly selected, euthanized, and their eyeballs enucleated. Specimens were fixed in 4% paraformaldehyde, dehydrated through graded alcohols, embedded in paraffin, and sectioned at 4 m. Selected sections were stained with hematoxylin-eosin (HE) to evaluate graft thickness and inflammatory cell infiltration under microscopy. Other sections underwent immunohistochemical detection using rabbit anti-rat SDF-1 antibody (ab9797, Abcam) and rabbit anti-rat CXCR4 antibody (ab124824, Abcam) as primary antibodies, and goat anti-rabbit antibody (ab97049, Abcam) as secondary antibody. The SP three-step method was employed with heat-mediated antigen retrieval and DAB chromogenic staining. PBS substitution for primary antibody served as negative control, and known positive sections served as positive controls. Cytoplasmic or nuclear brown-yellow staining indicated positive cells. Sections were examined and photographed at 400× magnification (OLYMPUS DIGITAL CAMERA, Japan). Five random fields per immunostained corneal section were analyzed using Image-Pro Plus software, and mean optical density was calculated as the semi-quantitative result.

1.5 Real-Time Quantitative PCR

On days 5 and 9 postoperatively, three rats from each group were randomly selected, and corneal grafts were harvested into 1 mL tubes. Total RNA was extracted using Trizol method. RNA concentration and A260/A280 ratio were measured using Nanodrop. RNA was reverse-transcribed to cDNA using a reverse transcription kit (Cat: RR037A,

Takara). qPCR was performed with rat GAPDH as internal control. Primer sequences were: GAPDH forward 5' -ACCACAGTCCATGCCATCAC-3' , reverse 5' -TCCACCACCCTGTTGCTGAT-3' ; SDF-1 forward 5' -CCCTAACCAGTTAGCTTCATCC-3', reverse 5'-GAGAAGCTCCAAAGCAAACC-3' ; CXCR4 forward 5' -TGAATGAGTGTCTAGGCAGGA-3' , reverse 5' -CACATTCTGGAGCGTTCAGT-3' . Reaction mixtures were prepared according to the qPCR kit (Cat: RR420A, Takara) instructions, and three-step real-time PCR was performed using a 7500 q-PCR system (ABI, USA) with the following cycling conditions: 95°C pre-denaturation for 30 s, followed by 40 cycles of 95°C denaturation for 5 s and 60°C annealing for 34 s. All samples were run in duplicate.

1.6 Statistical Analysis

Data were analyzed using IBM SPSS Statistics 20. One-way ANOVA was used to test for differences in SDF-1/CXCR4 mRNA expression among groups, with LSD test for pairwise comparisons. Kaplan-Meier survival curves were generated to compare graft survival rates.

Results

2.1 Clinical Observation of Rejection

Within 7 days postoperatively, all surgical groups exhibited mild transient edema and opacity due to surgical trauma and suture irritation, without obvious rejection. As shown in [Figure 1: see original paper], on day 5, corneal grafts in the autograft, allograft, and TobraDex groups showed no significant differences under microscopy, with minimal neovascularization around sutures and maintained transparency (Figures 1A-1C). From day 8 onward, grafts diverged: autograft corneas remained clear throughout the observation period without rejection, though extensive neovascularization developed around sutures and grafts (Figure 1D). Allograft corneas developed severe edema and opacity from day 8, with obscured iris patterns, barely visible pupil contours, and extensive neovascularization invading the central graft in some quadrants, progressing to rejection (Figure 1E) with a median survival time of 10 ± 0.36 days. TobraDex-treated grafts remained clear on day 9 with minimal perisutural neovascularization (Figure 1F), developing progressive edema and opacity only after day 20, with a median survival time of 24 ± 0.32 days. Kaplan-Meier analysis demonstrated a statistically significant difference in mean graft survival between the allograft and TobraDex groups (^{2} = 21.60, P < 0.001).

2.2 Histopathology and Immunohistochemistry

HE staining revealed clear corneal architecture without vessels or lymphocytic infiltration in normal controls ([Figure 2: see original paper]A, 2E). On day 5, autografts showed clear architecture with occasional inflammatory cells in the

stroma (Figure 2B), while allografts exhibited numerous inflammatory cells without evident vascular lumens (Figure 2C), and TobraDex grafts showed normal structure without significant vascularization or lymphocytic infiltration (Figure 2D). On day 9, autografts contained more inflammatory cells than on day 5 with evident stromal vascular lumens (Figure 2F). Allografts showed massive inflammatory infiltration, disorganized architecture, and prominent stromal vascular lumens (Figure 2G). TobraDex grafts displayed minimal vascular lumens and few inflammatory cells (Figure 2H).

Immunohistochemistry demonstrated low SDF-1 expression in normal corneas (Figures 2I, 2M). All transplant groups showed higher SDF-1 positivity than normal controls, with the strongest expression in allografts, particularly in the epithelium (strongly positive; Figures 2K, 2O). On day 9, epithelial SDF-1 positivity decreased in all groups compared to day 5, while stromal positivity increased slightly. CXCR4 showed minimal expression on occasional epithelial cells in normal corneas (Figures 2Q, 2U). On day 5, few positive cells appeared in the epithelium of autografts and allografts (Figures 2R, 2S). On day 9, autografts and TobraDex grafts showed scant positive cells (Figures 2V, 2X), while allografts exhibited numerous CXCR4-positive cells in both stroma and epithelium (Figure 2W). Semiquantitative analysis using Image-Pro Plus revealed that on day 5, allograft SDF-1 integrated optical density (IOD) (0.0174 ± 0.0010) was significantly higher than TobraDex grafts (0.0071 ± 0.0007, $P < 0.01$, [Figure 3: see original paper]A) and day 9 allografts (0.0921 ± 0.0017, $P < 0.01$). On day 9, allograft CXCR4 IOD (0.0449 ± 0.0047) was significantly higher than TobraDex grafts (0.0251 ± 0.0007, $P < 0.05$) and day 5 allografts (0.0225 ± 0.0038, [Figure 3: see original paper]B).

2.3 Real-Time Quantitative PCR Results

On day 5, SDF-1 mRNA expression was elevated in all transplant groups compared to normal controls, with the highest level in allografts ($P < 0.001$ vs. all other groups). This pattern persisted on day 9, though expression was lower than on day 5 in all groups ($P < 0.05$, [Figure 4: see original paper]A). CXCR4 mRNA expression on day 5 showed no significant differences among normal, autograft, and TobraDex groups, but was significantly increased in allografts ($P < 0.05$). On day 9, CXCR4 mRNA was elevated in all transplant groups versus normal controls, with the highest expression in allografts ($P < 0.01$). Expression was higher on day 9 than day 5 in all groups ($P < 0.01$ for autograft and allograft; $P < 0.05$ for TobraDex, [Figure 4: see original paper]B).

Discussion

Corneal transplant rejection is a complex immune response primarily mediated by T cells with participation of multiple immune cells and molecules. The immune response following corneal transplantation involves three main phases: sensitization, immune cell proliferation/activation, and effector stages [13-14].

Chemokines are small (8-10 kDa) proteins with structural similarity that directionally chemoattract specific cells. Chemokine receptors belong to the seven-transmembrane G protein-coupled receptor superfamily and are classified as CXCR, CCR, CR, and CX3CR based on their ligand specificity. CXCR4, a CXCR family member, is the specific receptor for SDF-1. SDF-1/CXCR4 binding activates both G protein-dependent and -independent pathways to mediate immune cell maturation and chemotaxis [3, 5, 15-16].

In corneal alkali burn models, SDF-1/CXCR4 expression has been shown to participate in corneal neovascularization [17-19]. Immunological studies demonstrate CXCR4 expression on lymphocytes, monocytes, and neutrophils, with SDF-1 playing crucial roles in their maturation and migration [20-23]. In hematopoietic stem cell transplantation, AMD3100-mediated SDF-1/CXCR4 blockade significantly improved host survival [24]. Similarly, blocking SDF-1/CXCR4 enhanced graft survival in pancreas, kidney, and artery transplantation [6-8, 25-26]. However, despite the unique anatomical position of the cornea and its distinct immune responses compared to other solid organs [10], SDF-1/CXCR4 remains unstudied in corneal transplantation.

This study is the first to investigate SDF-1/CXCR4 in rat corneal transplant rejection. We found significantly elevated SDF-1 and CXCR4 expression in rejecting allografts, which decreased with TobraDex treatment, suggesting SDF-1/CXCR4 involvement in the rejection process. Comparing days 5 and 9, SDF-1 levels peaked on day 5 and declined by day 9, indicating its primary role in early rejection. Immunohistochemical localization revealed SDF-1 predominantly in the epithelium on day 5, with CXCR4-positive cells also confined to the epithelium at this time point. By day 9, epithelial SDF-1 decreased while stromal expression increased, and CXCR4-positive cells became abundant in the stroma. These findings suggest that SDF-1 induces CXCR4+ cell maturation and chemotaxis to rejection sites, and that SDF-1/CXCR4 may mediate both epithelial and stromal rejection pathways. TobraDex treatment markedly reduced neovascularization compared to allografts. Combined with previous corneal alkali burn studies, we propose that SDF-1/CXCR4 also participates in graft neovascularization, disrupting corneal immune privilege and promoting rejection. Definitive mechanistic conclusions will require specific SDF-1/CXCR4 blockade or gene knockout models, representing a limitation of this study and future research direction for our group.

Conclusion

In summary, this study is the first to explore SDF-1/CXCR4 function in corneal transplantation, demonstrating that SDF-1/CXCR4 likely participates in early rejection after rat corneal transplantation. The mechanism may involve SDF-1 specifically inducing CXCR4+ cell maturation and chemotaxis toward the graft while promoting corneal neovascularization.

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Figure Captions

[Figure 1: see original paper] Corneal grafts after transplantation in different groups. A-F: Corneal grafts on days 5 and 9 after transplantation showing corneal opacity, neovascularization and edema; H: Corneal graft in Tobradex group on day 20; G: Rejection scores in the groups (n=10).

[Figure 2: see original paper] HE staining (A-H) and immunohistochemistry for SDF-1 (I-P) and CXCR4 (Q-X) of the corneal grafts on posttransplant day 5 and 9 (Original magnification: ×400). The inserts on the upper left corners in Q-X represent magnified images (×20) of the areas in red box.

[Figure 3: see original paper] Mean optical density (IOD) of immunohistochemistry SDF-1 (A) and CXCR4 (B) in different groups on days 5 and 9. *P<0.05 vs the other groups on day 9; **P<0.01 vs the other groups on day 5 and vs allograft group on day 9; &P<0.05 vs the other groups on day 9 and vs allograft group on day 5; #P<0.05 vs normal and Tobradex groups on day 5.

[Figure 4: see original paper] Relative mRNA expression levels of SDF-1 (A) and CXCR4 (B) in the groups on days 5 and 9 (n=3). *P<0.05 vs normal group on day 9; **P<0.01 vs the other groups on day 5 and vs allograft group on day 9; ##P<0.01 vs normal and TobraDex group on day 9 and vs allograft group on day 5; #P<0.05 vs normal and autograft group on day 9 and vs allograft group on day 5; @@P<0.01 vs TobraDex group on day 9; @P<0.05 vs TobraDex group on day 5 and allograft group on day 9; @@P<0.01 vs normal and autograft groups on day 5.

Note: Figure translations are in progress. See original paper for figures.