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Normalization of wound healing and stem cell marker patterns in organ-cultured human diabetic corneas by gene therapy of limbal cells

Mehrnoosh Saghizadeh [a, b, c, d], Christian M. Dib [d], William J. Brunken [e, f], Alexander V. Ljubimov [a, b, c, d, *]

[a] Eye Program, Board of Governors Regenerative Medicine Institute, Cedars-Sinai Medical Center, Los Angeles, CA, USA
[b] Department of Neurosurgery, Cedars-Sinai Medical Center, Los Angeles, CA, USA
[c] Department of Biomedical Sciences, Cedars-Sinai Medical Center, Los Angeles, CA, USA
[d] University of California Los Angeles, Los Angeles, CA, USA
[e] Center for Vision Research, Department of Ophthalmology, SUNY Upstate Medical University, Syracuse, NY, USA
[f] Department of Neuroscience and Physiology, SUNY Upstate Medical University, Syracuse, NY, USA

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ABSTRACT

Overexpression of *c-met* and suppression of *matrix metalloproteinase-10 (MMP-10)* and *cathepsin F* genes was previously shown to normalize wound healing, epithelial and stem cell marker patterns in organ-cultured human diabetic corneas. We now examined if gene therapy of limbal cells only would produce similar effects.

Eight pairs of organ-cultured autopsy human diabetic corneas were used. One cornea of each pair was treated for 48 h with adenoviruses (Ad) harboring full-length *c-met* mRNA or a mixture (combo) of Ad with *c-met* and shRNA to *MMP-10* and *cathepsin F* genes. Medium was kept at the limbal level to avoid transduction of central corneal epithelium. Fellow corneas received control Ad with *EGFP* gene. After additional 5 (*c-met*) or 10 days (combo) incubation, central corneal epithelial debridement with n-heptanol was performed, and wound healing times were determined microscopically. Corneal cryostat sections were immunostained for diabetic and putative limbal stem cell markers, $\alpha_3\beta_1$ integrin, nidogen-1, fibronectin, laminin γ3 chain, ΔNp63α, keratins 14, 15, and 17, as well as for activated signaling intermediates, phosphorylated EGFR, Akt, and p38.

Limbal c-met overexpression significantly accelerated healing of 8.5-mm epithelial wounds over EGFP controls (6.3 days vs. 9.5 days, $p < 0.02$). Combo treatment produced a similar result (6.75 days vs. 13.5 days, $p < 0.03$). Increased immunostaining vs. EGFP controls for most markers and signaling intermediates accompanied *c-met* gene or combo transduction.

Gene therapy of limbal epithelial stem cell compartment has a beneficial effect on the diabetic corneal wound healing and on diabetic and stem cell marker expression, and shows potential for alleviating symptoms of diabetic keratopathy.

1. Introduction

Diabetic retinopathy (DR) is the major complication of diabetes mellitus in the eye. It occurs both in type I (insulin-dependent) and type II (non-insulin-dependent) diabetes (Antonetti et al., 2012). Vision loss from diabetes is mainly due to the retinal changes but other parts of the eye are also affected. In the iris, it is neovascularization and neovascular glaucoma; in the lens, diabetic cataract; in the optic nerve, glaucomatous optic neuropathy (Nakamura et al., 2005; Blum et al., 2007; Helbig, 2007; Murtha and Cavallerano, 2007). Depending on disease duration and retinopathy stage, these symptoms are encountered in less than 30% of diabetic patients. The diabetic corneal disease includes recurrent erosions, delayed and incomplete wound healing, ulcers and edema, complications after vitrectomy and corneal surgery, and neuropathy/loss of corneal sensation; up to 70% diabetics have corneal problems (Schultz et al., 1981; Herse, 1988; Didenko et al., 1999; Wylegała et al., 2006; Chen et al., 2009; Bikbova et al., 2012;

* Corresponding author. Eye Program, Board of Governors Regenerative Medicine Institute, Cedars-Sinai Medical Center, 8700 Beverly Boulevard, AHSP-A8106, Los Angeles, CA 90048, USA.
E-mail address: ljubimov@cshs.org (A.V. Ljubimov).

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Table 1
Donor characteristics.

Case number	Age, years	Sex	DM type	Duration, years	Cause of death	Culture treatment
DR 11-20	62	M	NIDDM	10	Congestive heart failure	*c-Met*
DM 11-21	73	M	NIDDM	10	Pulmonary edema	*c-Met*
DM 11-28	71	F	NIDDM	8	Myocardial infarction	*c-Met*
DM 11-29	84	M	NIDDM	30	Congestive heart failure	*c-Met*
DM 12-19	79	M	NIDDM	Over 10	Hyperkalemic cardiac arrest	Combo
DM 12-21	84	F	NIDDM	20	Alzheimer's disease	Combo
DM 12-22	60	M	NIDDM	10	Cardiovascular disease	Combo
DM 12-23	71	F	NIDDM → IDDM	14 → 1	Anoxic encephalopathy	Combo

Combo, sh-cathepsin F + sh-MMP-10 + *c-met*. Arrows denote the number of years with a particular type of diabetes.

Calvo-Maroto et al., 2014). The most severe proliferative stage of DR cannot be recapitulated in animal models. We have previously validated human diabetic corneal organ culture system that reproduces wound healing dynamics and diabetic marker distribution (Kabosova et al., 2003), most probably due to the existence of epigenetic metabolic memory (Roy et al., 1990; Grant et al., 1998; Kowluru et al., 2013). We have further developed specific gene therapy that effectively reversed slow diabetic corneal epithelial wound healing and restored similar to normal expression of several diabetic markers and putative stem cell-associated proteins. This therapy was based on existing overexpression in diabetic corneas of matrix metalloproteinase-10 (MMP-10) and cathepsin F that we knocked-down using adenovirus (Ad)-driven shRNA transduction (Saghizadeh et al., 2010a, 2013). Additionally, *c-met* proto-oncogene, receptor of hepatocyte growth factor (HGF), was reduced in diabetic corneas and its levels were successfully restored using its Ad-driven overexpression (Saghizadeh et al., 2005, 2010b). The best normalizing effect on diabetic corneas was obtained using combination therapy approach, upon changing the levels of all three targets (Saghizadeh et al., 2013). As stem cells are necessary for epithelial wound healing, and diabetes-altered stem cell marker patterns became much closer to normal after gene therapy, we hypothesized that gene therapy could be also efficient if applied only to the limbus that harbors corneal epithelial stem cells (Lehrer et al., 1998; Lu et al., 2001; Rama et al., 2010; Amitai-Lange et al., 2014). It was, therefore, examined whether gene therapy of the limbus, to increase expression of c-met and knock-down MMP-10 and cathepsin F only in limbal cells, would restore putative stem cell marker expression and normalize wound healing in diabetic corneas. To ensure limbal involvement, healing of large 8.5 mm wounds was studied in organ-cultured corneas.

2. Methods

2.1. Human tissue and ethics statement

Postmortem diabetic human donor eyes were purchased from the National Disease Research Interchange (NDRI, Philadelphia, PA). NDRI has a human tissue collection protocol approved by a managerial committee and subject to National Institutes of Health oversight. This work was covered by an approved Cedars-Sinai Medical Center exempt IRB protocol EX-1055.

2.2. Organ culture, viral transduction and wound healing

Eight pairs of age-matched diabetic corneas (4 per group; Table 1) were organ-cultured in serum-free DMEM with insulin-transferrin-selenite (Kabosova et al., 2003). Ad harboring shRNA to *MMP-10* and *cathepsin F* genes (Saghizadeh et al., 2013) were obtained from Capital Biosciences (Gaithersburg, MD), and Ad overexpressing full-length *c-met* cDNA was engineered in the laboratory (Saghizadeh et al., 2010b). Control Ad (Capital Biosciences) drove enhanced green fluorescent protein (EGFP) expression. Virus incubation with corneas was performed for 48 h with medium level at the limbus to ensure that central corneas were not transduced (Saghizadeh et al., 2010a, 2010b, 2013). This was verified by EGFP expression in live corneas showing EGFP signal only in the limbal compartment (Fig. 1A). Details of the procedure have been published. After 5 days of additional incubation for Ad-c-met (Saghizadeh et al., 2010b) or 10 days for Ad-c-met and Ad-shRNAs (combo treatment; Saghizadeh et al., 2013), central corneal epithelial wounds were made for 1 min with round paper disks soaked in n-heptanol. Fellow corneas of each pair received Ad-EGFP and were also wounded. Wound closure was monitored microscopically as described (Saghizadeh et al., 2013), and expressed as the number of days to complete healing. In a pilot experiment, Ad-c-met transduced cornea and fellow Ad-EGFP control had a 5-mm wound made as described before (Saghizadeh et al., 2010a, 2010b, 2013). All other corneas had 8.5-mm wounds made to ensure limbal cell involvement in the healing process.

2.3. Immunostaining

Upon completion of the healing process, corneas were cut in half and embedded in O.C.T. compound (Sakura Finetek USA, Torrance, CA). Cryosections were immunostained using specific antibodies for a variety of markers (Table 2; Saghizadeh et al., 2013). These included proteins used for transduction (c-met, MMP-10, and cathepsin F), proteins with reduced expression in diabetic corneas

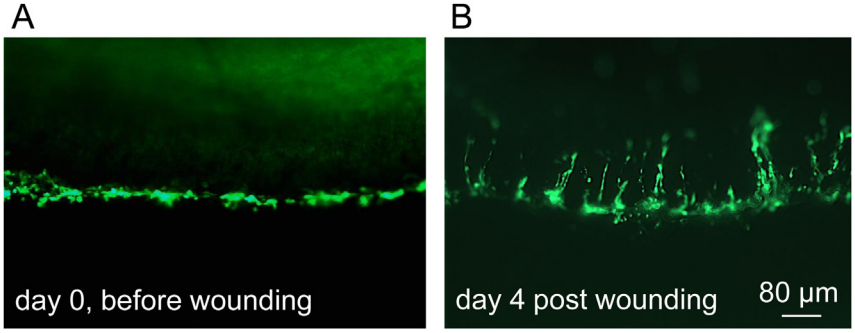

Fig. 1. Transduction of organ-cultured diabetic cornea with Ad-EGFP (live images). A, only limbal region is transduced (EGFP + cells, green). B, during healing of a large 8.5 mm wound, limbal cell migrate centripetally. Days are relative to wound healing study. e, epithelium, s, stroma. Bar = 80 μm. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)