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Short Communication

Promotion of corneal epithelial wound healing in diabetic rats by the combination of a substance P-derived peptide (FGLM-NH_2) and insulin-like growth factor-1

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Abstract

Aims/hypothesis. The healing of corneal epithelial wounds is often delayed in individuals with diabetes. The effect of the combination of a substance P-derived tetrapeptide (phenylalanine-glycine-leucine-methionine amide, or FGLM-NH_2) and insulin-like growth factor-1 (IGF-1) on corneal epithelial wound healing was investigated in rats with streptozotocin-induced diabetes.
Methods. The corneal epithelium of diabetic and non-diabetic rats was removed, and the animals were treated by the application of eye drops containing FGLM-NH_2 and IGF-1, or vehicle alone as a control, six times a day for 3 days. The area of the corneal epithelial wound was measured at various times up to 72 h after removal of the corneal epithelium.
Results. The rate of corneal epithelial wound healing was slower in diabetic rats treated with vehicle than in non-diabetic rats. However, the rate of wound closure in diabetic rats treated with FGLM-NH_2 and IGF-1 was markedly increased compared with that in diabetic rats treated with vehicle. The wound healing process seemed similar in normal rats and in diabetic rats treated with FGLM-NH_2 and IGF-1.
Conclusion/interpretation. The combination of FGLM-NH_2 and IGF-1 promotes corneal epithelial wound healing in diabetic rats, suggesting that such a treatment might prove effective in humans with diabetic keratopathy. [Diabetologia (2003) 46:839–842]

Keywords Diabetes, corneal epithelium, wound healing, substance P-derived peptide, insulin-like growth factor-1.

Various types of diabetic complication manifest in the eye. Diabetic retinopathy is one of the most serious sight-threatening complications of diabetes. However, the cornea is also affected in diabetic individuals. The clinical signs of diabetic keratopathy include persistent epithelial defects, superficial punctate keratopathy, and recurrent epithelial erosion. Both a loss of corneal sensation and reduced tear secretion [1] as well as abnormalities of the corneal epithelial basement membrane [2, 3] have also been described in humans or animals with diabetes. The cornea of individuals with diabetes could seem normal until the corneal surface is damaged, when a delay in epithelial healing becomes apparent. Such a delay in corneal epithelial wound healing is thought to underlie the pathology of diabetic keratopathy.

The biological characteristics of the cornea render it amenable to the study of epithelial wound healing, given that it lacks a vascular system and it is possible to distinguish reepithelialization from fibroblastic granulation. We and others have shown a delay in corneal epithelial wound healing in diabetic rats [4, 5]. The underlying basement membrane or a temporary matrix, as well as cell surface integrins, are important for the attachment of epithelial cells during corneal epithelial wound healing [6]. We previously showed

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Abbreviations: SP, substance P; FGLM-NH_2, phenylalanine-glycine-leucine-methionine amide.

that the administration of eye drops containing fibronectin or hyaluronan facilitates corneal epithelial wound closure in rats with streptozotocin-induced diabetes [5], indicating that the exogenous fibronectin or hyaluronan provides a functional matrix for epithelial cell migration.

We also recently showed that substance P (SP) and insulin-like growth factor-1 (IGF-1) synergistically promote corneal epithelial migration [7], with neither agent alone having an effect on this process. The combination of SP and IGF-1 also induced up-regulation of the expression of integrin α5 as well as phosphorylation of paxillin and focal adhesion kinase in corneal epithelial cells [8]. The combination of SP and IGF-1 therefore seems to affect the fibronectin-integrin signaling pathway during corneal epithelial migration. We further found that the minimum amino acid sequence of SP required for its synergistic effect with IGF-1 on corneal epithelial migration was phenylalanine-glycine-leucine-methionine amide (FGLM-NH_2) at the carboxyl terminus [9]. Treatment with the combination of FGLM-NH_2 and IGF-1 was thus as effective as was that with SP and IGF-1 in promoting corneal epithelial wound healing in rabbits. Furthermore, we showed that topical application of eye drops containing FGLM-NH_2 and IGF-1 was effective in the treatment of humans with neurotrophic keratopathy [10].

We investigated whether the combination of FGLM-NH_2 and IGF-1 promotes healing of the corneal epithelium in rats with streptozotocin-induced diabetes.

Materials and Methods

Male Sprague-Dawley rats (4 weeks old; body mass, ~100 g) were obtained from Japan SLC (Shizuoka, Japan). For induction of diabetes, 20 rats were deprived of food overnight and then injected in the tail vein with streptozotocin (70 $mg \cdot kg^{-1}$ of body mass) (Sigma, St. Louis, Mo., USA) in 0.01 mol/l citrate buffer. Control rats were injected with citrate buffer alone. Care and treatment of animals adhered to the Principles of Laboratory Animal Care (NIH publication no. 85-23, revised 1985).

Two weeks after injection of streptozotocin, the rats were anaesthetized with an intraperitoneal injection of pentobarbital, and one drop of topical oxybuprocaine was applied to each eye. The corneal epithelium was removed from each eye, from limbus to limbus, by scraping with a blunt scalpel blade. Beginning immediately after removal of the corneal epithelium, animals were treated six times a day for 3 days with eye drops (5 μl) containing either 1 mmol/l FGLM-NH_2 (Peptide Institute, Osaka, Japan) and IGF-1 (1 μg.ml-1) (Becton Dickinson, Bedford, Mass., USA) in phosphate-buffered saline (PBS) or PBS alone. Both eyes of each rat received the same treatment. The corneal epithelial defects were stained with 1 μl of 1% fluorescein and photographed both immediately after scraping and at various times up to 72 h thereafter. The area of each epithelial defect was measured on the photographs with a computer-assisted digitizer. The experiments were carried out in a double-blind manner to avoid any bias.

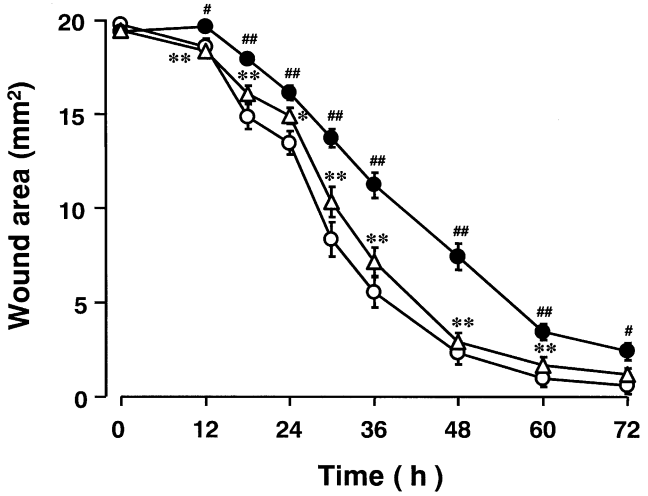

Fig. 1. Effect of the combination of FGLM-NH_2 and IGF-1 on corneal epithelial wound healing in diabetic rats. Wound healing was monitored for the indicated times after removal of the corneal epithelium in non-diabetic rats (*open circles*), diabetic rats treated with vehicle (*solid circles*), and diabetic rats treated with FGLM-NH_2 (1 mmol/l) and IGF-1 (1 μg/ml) (triangles) for 3 days. Data are means ± SEM of values from 12 to 16 eyes. #p<0.05, ##p<0.01 vs. non-diabetic rats; *p<0.05, **p<0.01 vs. diabetic rats treated with vehicle

Table 1. Body mass and blood glucose concentration of non-diabetic and diabetic rats

Group	Body mass (g)	Blood glucose (mg/dl)
Non-diabetic rats	187.4±3.8*	138.8±5.2*
Diabetic rats		
Vehicle	83.6±2.1	386.1±44.5
FGLM-$NH_2$and IGF-1	91.0±3.9	350.5±19.1

Data are means ± SEM of values from six to eight rats. * p<0.01 versus diabetic rats treated with either vehicle or the combination of FGLM-$NH_2$and IGF-1

After treatment for 3 days, the diabetic condition of rats was confirmed by measurement of body mass and of blood glucose concentration. The body mass and blood glucose concentration of streptozotocin-injected rats were smaller and 2.6 times greater, respectively, than those of vehicle-injected rats(p<0.01). There were no differences in these parameters between rats treated with the combination of FGLM-NH_2 and IGF-1 and those treated with PBS vehicle (Table 1).

Data are presented as means ± SEM and were compared between two groups by Student's t test. A p value of less than 0.05 was considered statistically significant.

Results

No difference in the size of the corneal epithelial defect immediately after wounding was apparent between diabetic and non-diabetic rats (Fig. 1). Epithelial healing was relatively slow during the first 12 h

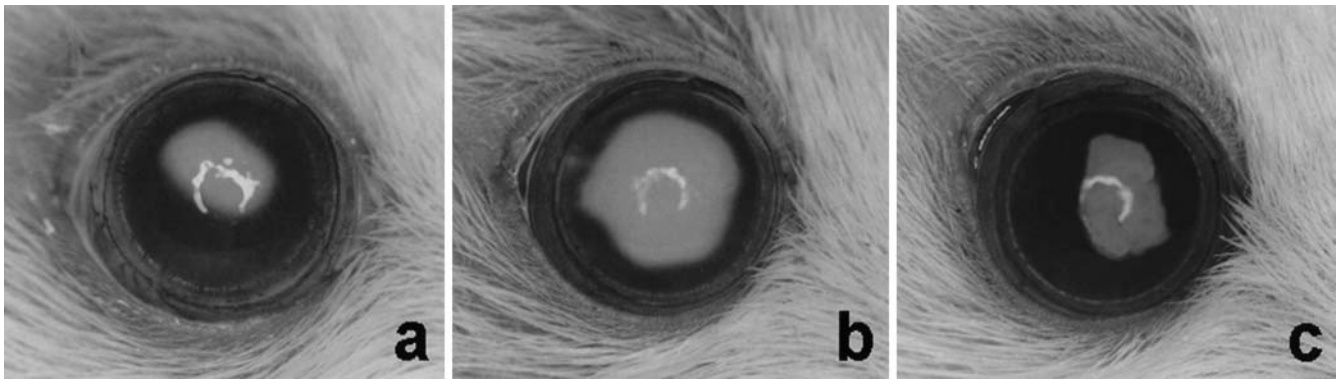

Fig. 2A–C. Photographs of fluorescein staining of the cornea 36 h after removal of the corneal epithelium in a non-diabetic rat (**A**), a diabetic rat treated with vehicle (**B**), and a diabetic rat treated with FGLM-NH_2 (1 mmol/l) and IGF-1 (1 μg/ml) (**C**)

after wounding, increased in rate from 12 to 48 or 60 h, and then slowed again thereafter in all three groups of rats (non-diabetic rats, diabetic rats treated with PBS, and diabetic rats treated with FGLM-NH_2 and IGF-1). However, the wound area of vehicle-treated diabetic rats was larger than that of non-diabetic rats 12 h after removal of the corneal epithelium. This difference in wound size between these two groups of rats remained significant throughout the 72-h study period, confirming that epithelial wound healing is delayed in diabetic rats.

Administration of the combination of FGLM-NH_2 and IGF-1 to diabetic rats increased the rate of wound healing, with the wound area in animals receiving this treatment being smaller than that in diabetic rats treated with vehicle at time points between 12 and 60 h after injury. The healing process in the diabetic rats treated with the combination of FGLM-NH_2 and IGF-1 seemed identical to that in non-diabetic rats.

We show typical photographs of epithelial defects stained with fluorescein 36 h after wounding (Fig. 2). The stained area of the non-diabetic rat eye is much smaller than that of the vehicle-treated diabetic rat eye. The stained area of the diabetic rat eye treated with FGLM-NH_2 and IGF-1 was also greatly reduced compared with that of the diabetic rat eye treated with vehicle and resembled that of the non-diabetic rat eye.

The mean healing rate during the middle phase (12 to 36 h) of wound closure in non-diabetic rats was 0.541±0.028 $mm^2 \cdot h^{-1}$, compared with values of 0.351±0.026 and 0.469±0.032 $mm^2 \cdot h^{-1}$ for diabetic rats treated with vehicle or with the combination of FGLM-NH_2 and IGF-1, respectively. The mean healing rate in vehicle-treated diabetic rats was significantly ($p<0.01$) smaller than that in non-diabetic rats, whereas the mean healing rate in diabetic rats treated with FGLM-NH_2 and IGF-1 was significantly ($p<0.01$) greater than that in vehicle-treated diabetic rats.

Discussion

We have shown that the administration of eye drops containing FGLM-NH_2 and IGF-1 promotes corneal epithelial wound healing in diabetic rats. The reason for the delay in corneal epithelial wound closure associated with diabetes is unclear. However, studies of the pathobiology of diabetic keratopathy have revealed changes in the interaction of corneal epithelial cells with the basement membrane, a thickening of the corneal basement membrane, abnormal attachment of the basement membrane to the stroma, and changes in the sites of hemidesmosome formation that are associated with this condition [1, 2, 3, 4]. These alterations might be related to the delay in corneal epithelial wound healing associated with diabetic keratopathy.

Substance P, a neuropeptide present in sensory nerves, plays an important role in corneal epithelial wound healing. We have previously shown that SP and IGF-1 synergistically facilitate corneal epithelial migration [7]. The mechanism of action of these agents seems to include up-regulation both of integrin expression and of signalling mediated by focal adhesion kinase and paxillin, events that are essential for the attachment of epithelial cells to extracellular matrix proteins [8]. Substance P is a member of the tachykinin family of peptides and comprises 11 amino acids. Only the SP carboxyl-terminal sequence FGLM-NH_2 is required, however, for the synergistic effect with IGF-1 on corneal epithelial migration [9]. In addition to showing the efficacy of eye drops containing both FGLM-NH_2 and IGF-1 in treatment of the persistent epithelial defects of individuals with neurotrophic keratopathy [10], we have obtained preliminary data that suggest that such treatment is effective in patients with diabetic keratopathy (unpublished observations).

Our demonstration of the efficacy of the combination of FGLM-NH_2 and IGF-1 for the treatment of corneal epithelial injuries suggests that a similar treatment also might promote reepithelialization of skin wounds. Chronic non-healing dermal ulcers are also a serious problem in diabetic patients. Further cellular and molecular investigations are needed to clarify the

mechanism by which the combination of FGLM-NH_2 and IGF-1 ameliorates the defect in wound healing associated with diabetes.

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