Enhancing Effects of Sericin on Corneal Wound Healing in Rat Debrided Corneal Epithelium

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The protein sericin is the main constituent of silk. We demonstrate the effects of sericin on corneal wound healing in rat debrided corneal epithelium. We also determined the effects of sericin on cell adhesion and proliferation in a human cornea epithelial cell line (HCE-T). Epithelium was removed from the corneas of rats with a BD Micro-Sharp™, and wounded corneas were dyed with a 1% fluorescein solution. The corneal wounds were monitored using a fundus camera TRC-50X equipped with a digital camera. The corneal wound of rats instilled with saline was approximately 10% healing at 12 h, and approximately 65% healing at 24 h after corneal epithelial abrasion. The corneal wounds of rats instilled with saline showed almost complete healing by 36 h after corneal epithelial abrasion. On the other hand, the corneal healing rate of rats instilled with sericin solution was higher than that of rats instilled with saline, and the corneal healing rate constant increased with increasing sericin concentration. In addition, the adhesion and proliferation of HCE-T cells treated with 0.01—0.5% sericin solutions were enhanced, reaching a maximum at treatments with 0.2 and 0.1% sericin solutions, respectively. The present study demonstrates that the instillation of sericin solution has a potent effect in promoting wound healing and wound-size reduction in rats, probably caused by increasing cell movement and proliferation.

Key words sericin; cornea; human cornea epithelial cell; corneal wound healing; cell growth promotion

The cornea is a highly specialized and unique organ in the human body. Because of its anatomic location, the cornea is continually subjected to abrasive forces and occasional mechanical trauma. Damage to the cornea can result in scarring or opacification, causing visual defects that compromise transparency and that can even lead to a complete loss of vision. The cornea mainly comprises three layers, including the epithelium, stroma and endothelium. The outermost layer, the corneal epithelium, is a self-renewing tissue, whose maintenance requires continuous cell proliferation throughout life, and is a protective barrier to the external environment. The corneal epithelial cell mass can be viewed as the result of three separate, independent phenomena.[1)] Thoft and Friend have termed these: X, the proliferation of basal epithelial cells; Y, the contribution to the cell mass by the centripetal movement of peripheral cells; and Z, epithelial cell loss from the surface. Corneal epithelial maintenance thus can be defined by the equation: $X+Y=Z$, which simply states that if the corneal epithelium is to be maintained, cell loss must be balanced by cell replacement.[2)] The corneal wound healing process is divided into three sequential and partially overlapping steps: epithelial cell loss from the surface (Z) reduces and eventually covers the wound surface (Y), while cell proliferation (X) provides cells to rebuild the tissue and tissue remodeling to restore the stratified epithelium.[3—9)] Therefore, an increase in the centripetal movement and cell proliferation of corneal epithelial cells acts to promote corneal wound healing.

Proteins such as fibroin and sericin are the main constituents of silk, with fibroin contributing 70 to 80% and sericin 20 to 30% of the total cocoon weight.[10)] When cocoons or raw silk are used for textiles, the sericin is mostly removed from the cocoon and disposed of unused. However, sericin has recently been investigated for its activities in biotechnological fields. It has been reported that sericin enhances the attachment and growth of mouse and human fibroblasts.[11,12)] Terada *et al.* found growth promotion in several human cell lines and mouse hybridomas when sericin was added to the culture media.[13)] Therefore, it is possible that sericin may be applied as eye drops for corneal wound repair.

In this study, we investigated the enhancing effects of sericin on corneal wound healing in the debrided corneal epithelium of rats. We also determined the effects of the sericin on cell adhesion and proliferation in a human cornea epithelial cell line (HCE-T).

MATERIALS AND METHODS

Animals and Reagents The rats used were 7-week-old male Wistar rats. They were housed under standard conditions (12 h/d fluorescent light (07:00—19:00), 25 °C room temperature) and allowed free access to a commercial diet (CE-2, Clea Japan Inc., Tokyo, Japan) and water. All procedures were performed in accordance with the regulations of the Kinki University School of Pharmacy Committee for the Care and Use of Laboratory Animals and the Association for Research in Vision and Ophthalmology resolution on the use of animals in research. Pure Sericin™ (30 kDa) was obtained from Wako Pure Chemical Industries (Osaka, Japan). All other chemicals used were of the highest purity commercially available.

Instillation of Sericin Solutions in Rats The sericin solutions used in this study were prepared by adding Pure Sericin™ to saline (pH 6.5—7.5). Five microliters of saline or sericin solution were instilled into the eyes of rats five times a day (9:00, 12:00, 15:00, 18:00, 21:00) after corneal abrasion. The eyes were kept open for about 1 min after instillation to prevent the sericin from overflowing.

Corneal Abrasion in Rats The rats were anesthetized

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with pentobarbital (30 mg/kg), and a 3.0-mm-diameter circle was outlined in the center of the cornea with a disposable dermatological skin punch (BIOPSY PUNCH, Kai Industries Co., Ltd., Gifu, Japan). The encircled corneal epithelium was removed with a BD Micro-Sharp™ (blade 3.5 mm, 30°, Becton Dickinson, Fukushima, Japan). The areas of removed corneal epithelium were followings: saline, 7.24±0.29; 1% sericin, 7.10±0.49; 5% sericin, 7.20±0.36; 10% sericin, 7.29±0.05 (mm^2, means±S.E. of 3—5 independent rat corneas).

Image Analysis for Corneal Wound Healing in Rats The corneal area from which the epithelium was removed was dyed with a solution containing 1% fluorescein (Alcon, Tokyo, Japan) and 0.4% Benoxil (Santen Pharmaceutical Co., Ltd., Osaka, Japan).[14] Changes in the corneal wound area were monitored using a TRC-50X (Topcon, Tokyo, Japan) equipped with a digital camera (EOS Kiss Digital N, Canon Inc., Tokyo, Japan)[15]; wound area was analyzed with image analyzing software Image J.[16] Corneal wound healing (%) was calculated by the following Eq. 1:

$$\text{corneal wound healing (\%)} = (\text{wound area}_{0\,\text{h}} - \text{wound area}_{12,\,24\text{ or }36\,\text{h}})/\text{wound area}_{0\,\text{h}} \times 100 \quad (1)$$

The rate of corneal wound healing is represented by the corneal wound healing rate constant (k_H, h^{-1}). The k_H over the period 0—36 h after corneal epithelial abrasion was calculated from the following Eq. 2:

$$H_t = H_\infty \cdot (1 - e^{-k_H \cdot t}) \quad (2)$$

where t is time (0—36 h) after corneal abrasion, and H_∞ and H_t are the percentages of corneal wound healing (%) at time ∞ and t, respectively.

Cell Culture and Treatment The immortalized human corneal epithelial cell line (HCE-T) developed by Araki-Sasaki *et al.*[17] was used in this study. HCE-T cells were cultured in Dulbecco's modified Eagle's medium/Ham's F12 (GIBCO, Tokyo, Japan) containing 5% (v/v) heat-inactivated fetal bovine serum and 0.1 mg/ml streptomycin and 1000 IU/ml penicillin (GIBCO, Tokyo, Japan). In the cell adhesion experiment, sericin treatment was carried out by seeding HCE-T cells (1×10^4 cells) into culture medium containing 0 (control), 0.01, 0.05, 0.1, 0.2, 0.3, 0.4, 0.5 or 1.0% sericin in 96-well microplates (IWAKI, Chiba, Japan), and incubating the cells under humidified air containing 5% CO_2 at 37 °C for 12 h. In the cell growth experiment, sericin was added to cell cultures 1 d after seeding (1×10^4 cells) by changing to culture medium containing 0 (control), 0.01, 0.05, 0.1, 0.2, 0.3, 0.4, 0.5 or 1.0% sericin. The cells were then incubated under humidified air containing 5% CO_2 at 37 °C for 24 h. After sericin treatment, TetraColor One (SEIKAGAKU Co., Tokyo, Japan) was added, and the absorbance (Abs) at 490 nm was measured. Cell adhesion and growth were calculated by TetraColor One according to the manufacturer's instructions. Cell proliferation is represented by the following Eq. 3:

$$\text{cell adhesion or growth (\%)} = \text{Abs}_{\text{sericin treatment}}/\text{Abs}_{\text{control}} \times 100 \quad (3)$$

Statistical Analysis All data are expressed as the means±standard errors (S.E.). Statistical differences were evaluated by one-way analysis of variance (ANOVA) followed by Dunnett's multiple comparison. p values less than 0.05 were considered significant. The number of experiments performed in duplicate is given in the figure legends.

RESULTS

Effect of Sericin Solution Instillation on Corneal Wound Healing in Rats Figures 1 and 2 show images after corneal epithelial abrasion (Fig. 1) as documented by a TRC-50X equipped with a digital camera, and corneal wound healing levels (Fig. 2) of rat eyes instilled with 1, 5 and 10% solutions of sericin. The corneal wounds of rats instilled with saline were healed by approximately 10% healing at 12 h after abrasion, and by approximately 65% after 24 h. The corneal wounds of rat eyes instilled with saline were almost entirely healed 36 h after corneal epithelial abrasion. On the other hand, the corneal healing rates in rat eyes instilled with sericin solutions were faster than in the case of saline instillation, and the rate constants increased with increasing sericin concentrations (Table 1). The corneal wounds of rat eyes in-

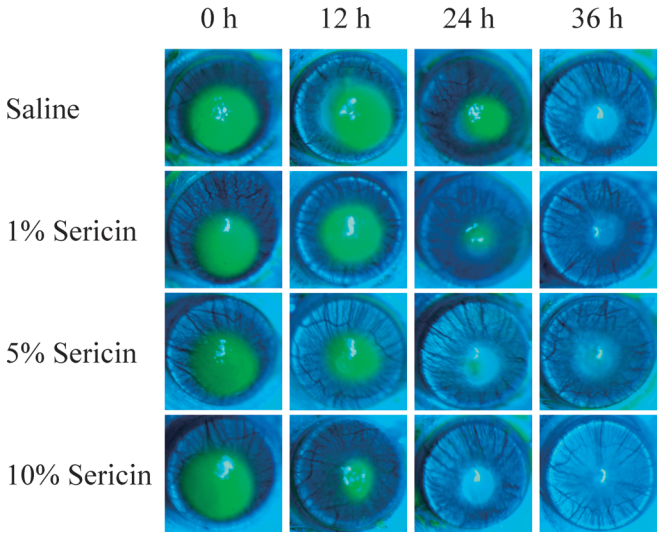

Fig. 1. Corneal Images of Rats with or without the Instillation of Sericin Solutions

The corneal epithelium was removed with a BD Micro-Sharp™, and the resulting corneal wounds were dyed with 1% fluorescein solution. The wounds were monitored using a TRC-50X (Topcon, Tokyo, Japan) equipped with a digital camera. Saline or sericin solutions were instilled into the eyes of rats five times a day.

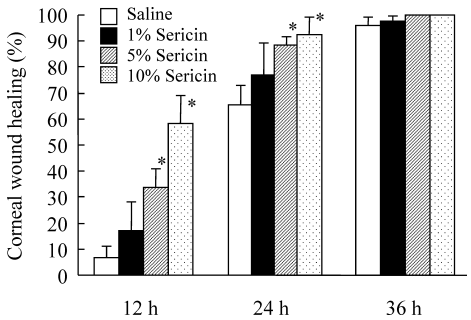

Fig. 2. Effect of Sericin Solutions on Corneal Wound Healing in Rat Eyes

The corneal epithelium was removed with a BD Micro-Sharp™, and the resulting corneal wounds were dyed with 1% fluorescein solution. The wounds were monitored using a TRC-50X equipped with a digital camera, and analyzed with Image J software. Corneal wound healing (%) was calculated according to Eq. 1 (see Materials and Methods). Saline or sericin solutions were instilled into the eyes of rats five times a day. The data are presented as means±S.E. of 3—5 independent rat corneas. $*p<0.05$, *vs.* saline-instilled rat.

Table 1. Rate of Corneal Wound Healing in Rats with or without the Instillation of Sericin Solutions

	Corneal wound healing rate constant ($\times 10^{-2}$/h)
Saline	0.56±0.53
1% sericin	1.44±0.82
5% sericin	2.09±0.80
10% sericin	6.83±2.23*

The rate of corneal wound healing was calculated according to equation 2 (see Materials and Methods). Saline or sericin solutions were instilled into the eyes of rats five times a day. The data are presented as means±S.E. of 3—5 independent rat corneas. * $p<0.05$, *vs.* saline-instilled rat.

stilled with 10% sericin solution were healed by approximately 60% 12 h after corneal epithelial abrasion, and the wounds showed almost complete healing 24 h after abrasion. The corneal healing rate constant of rat eyes instilled 10% sericin solution was approximate 12-fold that of rat eyes instilled with saline.

Effect of Sericin Solution on the Adhesion and Proliferation of HCE-T Cells Figures 3 and 4 show the changes in the adhesion and proliferation of HCE-T cells induced by treatment with 0.01—1.0% sericin solution. Both cell adhesion and proliferation were increased by treatment with 0.01—0.5% sericin solution, reaching a maximum at 0.2 and 0.1% sericin solution, respectively, and subsequently decreasing. The adhesion and proliferation levels of 1.0% sericin-treated HCE-T cells showed no significant differences from those of control HCE-T cells.

DISCUSSION

The corneal wound repair process involves cell adhesion, migration, proliferation, matrix deposition and tissue remodeling.[18] Many of these biological processes are mediated by growth factors, cytokines and other mediators released in injured tissues or cells.[19] These growth factors have been recognized as important mediators of proper wound repair,[20] and treatment with growth factors such as platelet-derived growth factor-BB, recombinant human epidermal growth factor and fibronectin has been shown to be beneficial for patients with chronic pressure ulcers or non-healing diabetic ulcers.[21—24] However, these autologous serum eye drops have problems in terms of safety and stability. Therefore, a potent corneal wound-healing agent for human corneal wounds that avoids these problems is highly anticipated. In this study, we investigate the healing effect of sericin on corneal wounds in rat debrided corneal epithelium.

In the rat corneal wound model, rat eyes instilled with saline showed approximately 10% healing at 12 h, and approximately 65% healing at 24 h after corneal epithelial abrasion. The corneal wounds of rats instilled with saline were almost entirely healed at 36 h after corneal epithelial abrasion. The centripetal movement and proliferation of corneal epithelial cells are important for the healing of corneal wounds in rat exfoliated corneal epithelium. In general, it is known that epithelial cells from the surface reduce and eventually cover the wound surface, with cell proliferation providing cells to rebuild the tissue and tissue remodeling to restore the stratified epithelium.[3—9] In addition, cell proliferation starts approximately 12 h after corneal epithelial abrasion.[25] Therefore, the reduction in the size of the wound may be due to cell movement during the 12 h after corneal epithelial abrasion, followed by cell proliferation. Thoft and Friend[2] showed that corneal epithelial maintenance can be defined by the equation: X+Y=Z (X, the proliferation of basal epithelial cells; Y, the contribution to the cell mass by centripetal movement of peripheral cells; and Z, epithelial cell loss from the surface). The corneal wounds in this study are caused by an enhancement of Z. Therefore, cell movement and proliferation are related to wound healing in this rat eye model. The rate of corneal wound healing in rat eyes instilled with sericin solution is higher than in eyes instilled with saline, and the rate constant increases with increasing sericin concentration. The corneal wounds of rat eyes instilled with 10% sericin solution showed 60% healing 12 h after corneal epithelial abrasion, and were almost entirely healed 24 h after abrasion. In addition, the corneas of rat eyes instilled with 10% sericin solution showed no observable neovascularization, and the corneal wound healing effect of 10% sericin in this rat eye model was significantly greater than that of 0.1% hyaluronic acid, which is frequently used in clinical treatment (k_H of 0.1% hyaluronic acid, $1.25\pm0.60\times0^{-2}$/h, means±S.E. of 3 independent rat corneas). These results suggest that sericin increases cell movement and proliferation, and indi-

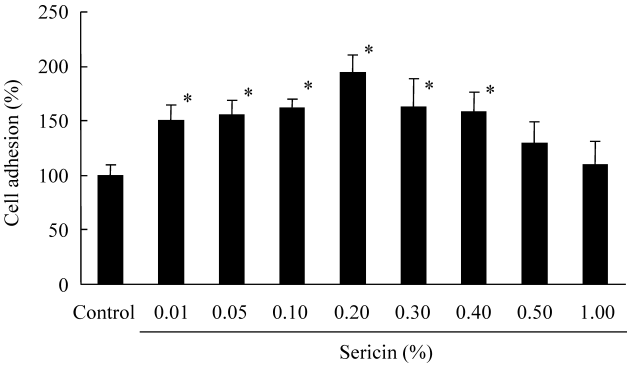

Fig. 3. Effect of Sericin on the Adhesion of HCE-T Cells

HCE-T cells were cultured in Dulbecco's modified Eagle's medium/Ham's F12 containing 5% (v/v) heat-inactivated fetal bovine serum, 0.1 mg/ml streptomycin and 1000 IU/ml penicillin. Cell growth was calculated by TetraColor One. The amount of cell adhesion was according to eq. 3 (see Materials and Methods). The data are presented as means±S.E. of 18—20 experiments. * $p<0.05$, *vs.* control HCE-T cells.

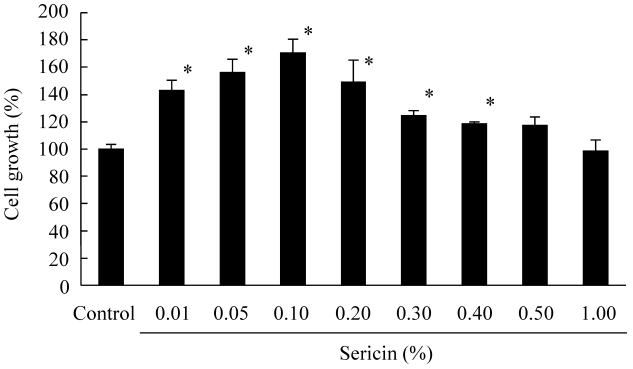

Fig. 4. Effect of Sericin on the Growth of HCE-T Cells

HCE-T cells were cultured in Dulbecco's modified Eagle's medium/Ham's F12 containing 5% (v/v) heat-inactivated fetal bovine serum, 0.1 mg/ml streptomycin and 1000 IU/ml penicillin. Cell growth was calculated by TetraColor One. The rate of cell growth was calculated according to Eq. 3 (see Materials and Methods). The data are presented as means±S.E. of 5—25 experiments. * $p<0.05$, *vs.* control HCE-T cells.

cate that sericin might provide an effective and safe drug for corneal wound healing.

It is important to confirm the mechanisms by which sericin promotes corneal wound healing, and it is important to investigate the effects of sericin on human corneal wounds. Terada *et al.* have found that sericin promotes the growth of several human cell lines and mouse hybridoma lines when it is added to the culture media.[13] In this study, we also show that sericin increases the adhesion and growth of HCE-T cells. Taken together, the instillation of sericin solution may cause an increase in cell adhesion and proliferation, resulting in the promotion of corneal wound healing. Thus the instillation of sericin solution may be effective for the treatment of human corneas.

In this study, the adhesion and proliferation of HCE-T cells reached a maximum when treated with 0.1—0.2% sericin solution; the levels of adhesion and proliferation of 1.0% sericin-treated HCE-T cells did not differ significantly from those of control HCE-T cells. On the other hand, the instillation of high concentration sericin solutions (1—10%) promoted enhanced wound healing in the corneal wound rat model. It is known that the concentration drugs administered in eye drops is diluted to approximately 20% by lacrimal fluids, and that the components of eye drops are excreted though the nasolacrimal duct into the mouth.[26] Thus, our findings suggest that the optimum concentration of the sericin solutions in the *in vivo* instillation experiment, which involves a short residence time, is higher than in the *in vitro* experiment. Further studies are needed to determine the precise mechanisms of corneal epithelial cell proliferation and the residence time of sericin following instillation. In addition, it is also important to clarify the effects of sericin on corneal wound healing in diabetic keratophathy, a condition characterized by slow healing or loose adhesion of the corneal epithelium after corneal wounding in diabetic patients, since diabetic keratophathy is experienced by 50% or more of all diabetic patients. Therefore, we are now investigating the effects of sericin on corneal wound healing in diabetic ulcers using the Otsuka Long-Evans Tokushima Fatty rat, a model of human type 2 diabetes.[27]

In conclusion, the present study demonstrates that the instillation of sericin solutions has the potential to promote wound healing and wound-size reduction in rat eyes, probably as a result of increased cell movement and proliferation. We conclude that the topical application of sericin in a safe and stable formulation enhances healing and reduces the size of corneal wounds in rat eyes. These findings provide significant information for designing further studies to develop potent corneal wound-healing drugs.

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