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Reversal of silver sulfadiazine-impaired wound healing by epidermal growth factor

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Abstract

Silver sulfadiazine (Ag-SD) is a useful antibacterial agent for wound treatment. However, recent findings indicate that the compound delays the wound-healing process. That delay may be reversed by treatment with growth factors. The purpose of this study, was to evaluate the cyto-protective effect of epidermal growth factor (EGF) against Ag-SD treated keratinocytes and to investigate the reversibility of the impaired wound-healing process by the co-supplementation of EGF. Four types of drug-loaded collagen sponge dressings with different concentrations of Ag-SD, EGF and Ag-SD + EGF were prepared. An immortalized keratinocyte, HaCaT cells, were cultured in 35-mm Petri-dish using Dulbecco's Modified Eagle's Minimal Essential Medium (DMEM) with 10% FBS. Cultures were treated with the samples submerged, and viabilities of cultures were evaluated using MTT assay. The wound heal efficacy was evaluated in a partial thickness burn mouse model. Cells treated with EGF showed a cyto-protective effect on 1% Ag-SD treated cells with significant increase in viable cell numbers at concentrations ranging from 1 to 50 μg/ml. The cytotoxicity of Ag-SD impaired wound healing, while the addition of EGF could reverse the impairment. This evidence suggests that EGF is a useful agent in the retardation of wound healing caused by Ag-SD. Therefore, a drug delivery system containing both EGF and Ag-SD, such as that used in the study, may be clinically relevant.

Keywords: Antibacterial; Growth factors; Keratinocyte; Cytotoxicity; Wound healing; Collagen

1. Introduction

Infection is one of the most frequent complications of the wound-healing process. It has been indicated that the increased inflammation leads to an overproduction of IL-8 and other inflammatory cytokines that inhibit keratinocyte replication disturb new matrix formation and retard wound closure [1,2]. Infection control is very important not only in the prevention of secondary infection but also in maintaining a proper wound-healing process. Although the use of topical antimicrobial agents is essential in the establishment of the bacterial balance in contaminated wounds, it has been associated with delayed healing of wounds in which the processes of skin cell proliferation and collagen deposition play a primary role [3].

Ag-SD has been a standard treatment for burns over the past three decades since Fox first synthesized silver sulfadiazine from silver nitrate and sodium sulfadiazine for an increased potency and negligible adverse effects including minimal pain on application in 1968 [4]. Ag-SD was reported to be particularly effective as a topical antibacterial agent for the control of *Pseudomonas* infection in burns [5]. We previously reported the cytotoxic effect of Ag-SD on HaCaT cells and delayed epithelialization process in a second-degree burn animal model [6]. It has been proposed that abnormal wound

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healing may result from insufficient or delayed production of growth promoting factors and their receptors. Therefore, it may be possible to reverse impaired wound healing by the exposure of cells in regenerating tissues to optimal levels of growth factors [7]. Furthermore, the literature suggests that sustained exposure to growth factors (e.g., epidermal growth factor (EGF)) may be necessary for proper wound healing [8–10]. Formulation of EGF in a simple, water-soluble vehicle that does not adequately sustain its release may not stimulate the rates of epithelialization and wound closure. Indeed, Brown et al. [11] have shown that EGF could not stimulate epidermal regeneration of the wounds when applied for 5 min in saline solution vehicle while EGF significantly stimulated epidermal regeneration when it was applied to partial thickness incisions in creams that released EGF for prolonged periods. In addition, recent studies indicated that absorption of silver from burn wounds lead to silver toxicity after a topical application of 1% Ag-SD cream [12]. These observations indicated that both EGF treatment for a prolonged period and limitation of the delivery of silver may be necessary for an optimal wound-healing effect. Unfortunately, however, such a delivery system for EGF and silver has not been developed. Therefore, the objective of this study was to prepare a topical delivery system for EGF and Ag-SD, and to evaluate the effectiveness. In this study, we were particularly interested in the feasibility of a collagen sponge wound dressing which can adequately release EGF and Ag-SD, since such a dressing formulation may be clinically applicable.

2. Materials and methods

2.1. Materials

Recombinant human EGF was provided by Daewoong Pharmaceuticals (Seoul, Korea). Ag-SD was donated from Dong Wha Pharmaceuticals (Seoul, Korea). All other ingredients were used as received. Duoderm™ (Convatec Co. Princeton, NJ), an occlusive dressing was used as a control formulation and Coban™ (3M Co., St. Paul, MN), a self-adherent wrap was used to prevent the detachment of dressings during experiment.

2.2. Collagen sponge preparation

The sponges were prepared using collagen acid extracted from young pig skin. Briefly, homogenized skin was dissolved in the solution of HCl and acetic acid, and collagen was fractionally precipitated by 5 M NaCl solution. The collagen preparation was diluted to obtain final concentration of collagen stock solution of 1%. The stock of 3.8 g was evenly distributed in 35-mm petridish, frozen at −70 °C and freeze-dried. Cross-linking of the collagen sponge was carried out by dipping a sponge in 5-ml hexamethylene diisocynate (1% in methanol) at room temperature for 10 min. Subsequently, the preparation was washed twice with methanol and water, respectively, and the freeze-dried sponge was stored at –20 °C until used. The size of resulting sponges was usually about 30 mm in diameter. When it was necessary to incorporate Ag-SD and/or EGF into collagen sponge, the sponge was submerged in 3 ml of sample solutions containing Ag-SD and/or EGF for 30 min and freeze-dried. The details of the sample solutions, collagen sponge weight before and after drug loading, and fractions of drug in sponge are summarized in Table 1.

2.3. Effect of Ag-SD and EGF on cell proliferation

Human HaCaT keratinocytes (kindly provided by Dr. N.E. Fusenig, DKFZ, Heidelberg, Germany) were incubated in 2-ml Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum (Gibco, Gaithersburg, MD) [13]. The cultures were maintained up to 80% confluence in 35-mm petridish before treating with Ag-SD and EGF. Samples of collagen sponge disk (0.2 cm^2) were prepared by soaking in different concentrations of Ag-SD and/or EGF solutions and freeze-dried. Before usage, the sponges

Table 1
Fraction of drug in collagen sponge treated with Ag-SD and EGF

Sample solution[a]	Dry collagen sponge (mg)	Dry collagen sponge after drug loading (mg)	Fraction of drug in sponge[b]
Ag-SD 0.5%	91.2±4.1	127.7±26.5	0.40±0.32
Ag-SD 1%	88.1±1.4	180.8±41.5	1.05±0.48
Ag-SD 2%	88.8±1.3	161.8±13.0	0.82±0.17
Ag-SD 1% +EGF 1 μg/ml	87.8±3.3	148.5±51.2	0.69±0.57
Ag-SD 1% +EGF 2 μg/ml	86.9±1.8	175.5±70.7	1.02±0.85
Ag-SD 1% +EGF 3 μg/ml	87.9±1.8	124.5±30.7	0.42±0.35

Each data represents mean±standard deviation from the triplicate samples.
[a]Collagen sponges of 30-mm diameter were submerged in 3 ml of sample solutions for 30 min and freeze-dried.
[b]Fraction = (weight of dry sponge after drug-loading−weight of dry sponge before drug-loading)/weight of dry sponge before drug-loading.

were sterilized by γ-irradiation. The sterile sponges of 30-mm diameter were placed in 6-well tissue culture plate (NUNC, Newbury Park, CA), seeded with 0.8×10^5 HaCaT cells per sponge and cultured in 5% CO_2 and 95% humidity for 1 week. Cell viability was assessed by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) staining method [14].

2.4. Evaluation of wound-healing property

Deep second-degree burn circular wounds (1-cm diameter) were prepared on the back skin of ICR mice of 5 weeks old. On the dorsal side, two sites of burn wound were induced to each mouse by applying 85 °C water for 10 s. After 24 h, the dead epidermis layer was scrapped off with curette. Specified dressing formulations were applied for 96 h.

Since the wound-healing process is dependent on the degree of absorption of wound exudates and occlusion, a non-adherent dressing, Duoderm^{TM} (Convatec Co., NJ, USA) was employed as a control dressing. After 96 h of the induction of the burn wound, dressings have been removed and the wound sites were investigated in terms of the sign of infection, the degree of epidermal regeneration, the exudates and the condition of the removed dressing. Subsequently, the degree of wound exudates, the sign of infection and the process of epithelialization and wound closure rate were evaluated at predetermined time intervals until the complete wound closure.

2.5. Histological evaluation

The skin specimens were excised and processed for histological evaluation either immediately after the removal of the dressing (i.e., 96 h after the induction of burn wound) or after the complete wound closure. At the time of the removal of the dressing, the burn wound still remained moist and the skin appeared viable as evidenced by the fact that healing process was apparently on-going in the specimens. Presence of inflammatory cells and degree of epidermal regeneration were investigated in the specimens obtained in occlusive dressing only, Ag-SD or Ag-SD + EGF treatment by using Olympus microscope (model BX41, Japan) and image recording equipment (models DP11 and PM10SP, Japan).

2.6. Statistical analysis

At least, triplicate experimental values were averaged for the evaluation of the wound closure rate, and data were expressed as mean ± standard deviation. Error bars in figures represent the standard deviation. Student's *t*-test was applied for the calculation of *p*-values to verify the difference between experimental data. For statistical analysis including standard deviation and significance of difference (*p*-value), statistics packages of SigmaStat^{TM} and SigmaPlot^{TM} from SPSS Inc. were used. The cases of *p*-value less than 0.05 were considered statistically different.

3. Results

We noted dramatic differences in wound closure were observed for formulations of 1% Ag-SD cream with EGF (4 μg/cm^2) in a second-degree burn animal model. EGF + Ag-SD-treated site showed complete wound closure in 7 d after the induction of a burn wound, while the burn wound treated with Ag-SD alone remained open (Fig. 1). Based on this finding, the effect of EGF on skin cells exposed to Ag-SD has been investigated in vitro, and the application of collagen sponge with Ag-SD and EGF as a wound dressing was evaluated.

The general appearance and scanning electron micrograph (SEM) of collagen sponge are depicted in Fig. 2. The sponge was fabricated in a circular disk with 30-mm diameter and 2-mm thickness. The pore size of the sponge was 50 to 100 μm. Interconnected channels of the sponge were readily apparent in SEM. Water absorption capacity of the sponge was from 500% to 700% of dry weight. The release rate of Ag-SD from the collagen sponge with 1% Ag-SD loaded was 79.1 μg/cm^{2}h. To increase the solubility of Ag-SD, *N*-(2-hydroxyethyl)piperazine (HEPES) buffer solution of 10 mM containing 140 mM KCl was employed as a release medium for Ag-SD. The solubility of Ag-SD increased up to 871.1 ± 6.6 μg/ml, compared to 8.3 ± 0.1 μg/ml in water.

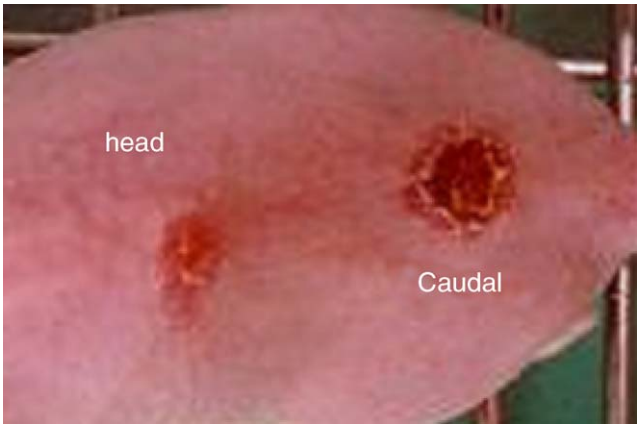

Fig. 1. Comparison of wound closure between EGF + Ag-SD and Ag-SD in 7 d after the induction of burn wound (i.e., deep second-degree burn: 85 °C × 10 s burn) in hairless mice. Key: head, Ag-SD 1% cream; caudal, Ag-SD 1% cream + EGF (4 μg/cm^2) in hydrogel of 20% gelatine (area: 1 cm^2, 4 μg EGF /50 mg of 20% gelatin /wound). EGF in 20% gelatin gel was applied on the treated skin area with a special application device, was fixed on the back skin of a mouse.

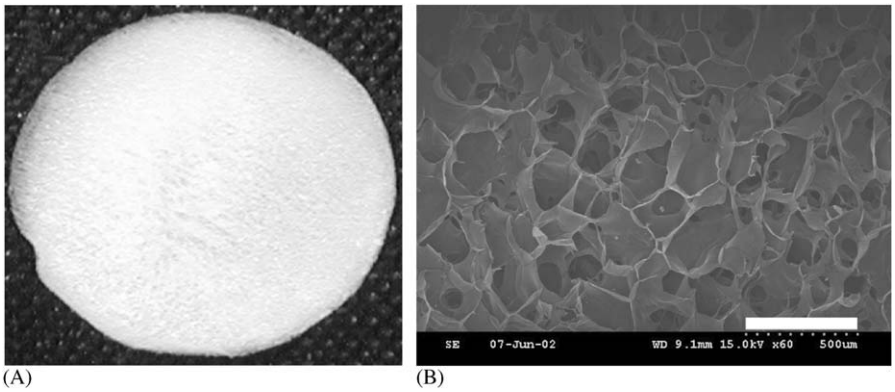

Fig. 2. General appearance (Panel A) and SEM micrography (Panel B) of collagen sponge. Scale bar represents 500 μm.

Mechanical strength and resistance against collagenase digestion were augmented by chemical cross-linking (data not shown).

Fig. 3 shows the effects of simultaneous supplementation of EGF and Ag-SD on viabilities of cultured HaCaT cells. Supplementation of Ag-SD (100 μg/ml) to culture media resulted in the reduced cell viability after 1 week of culture. However, the viability of cultured cells treated with Ag-SD was restored by EGF in a dose dependent manner (1 to 50 μg/ml). In other words, EGF-supplementation offset the loss of viability generated by Ag-SD, and the higher concentration of EGF in media recovered the viability loss more. In the case of 10 μg/ml of Ag-SD supplementation, cell viability was recovered up to ca. 90% of that of control culture without Ag-SD supplementation. The results indicate that EGF may have a cyto-protective function against Ag-SD in a cultured HaCaT cell line.

The cyto-toxicity of Ag-SD is also dose-dependent (data not shown). In Fig. 3, higher dosage of Ag-SD required more EGF supplementation to attain to a similar level of recovery of cell viability. For example, 10 and 50 μg/ml of EGF were needed, respectively, to overcome the toxic effect of 1 and 10 μg/ml of Ag-SD to a similar degree (ca. 90% viability). In addition, the cyto-protective effect of EGF on cells exposed to 100 μg/ml Ag-SD is less apparent, compared with those exposed to the lower concentration. It is possible that cell damage induced by the concentration over 100 μg/ml of Ag-SD may overwhelm cyto-protective capacity of EGF.

The underlying mechanism for the protective effect of EGF was not directly investigated in this study. However, it has been proposed that cells activated by growth factors either take up less Ag-SD or are more resistant to the direct cytotoxic effects of this drug [15]. Therefore, these secondary effects of EGF may participate in the reduced cellular damage created by Ag-SD.

Wound closure was tested for 2% Ag-SD collagen sponges containing 2 μg/cm^2 of EGF after 7 d of

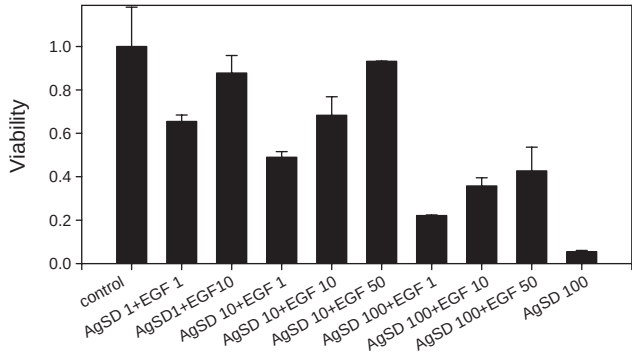

Fig. 3. Cyto-protection by EGF against cyto-toxicity of Ag-SD. HaCaT cells impaired by Ag-SD could regain the cell viability with co-supplementation of Ag-SD and the enhancement of viability was dose-dependent manner. Also, the higher Ag-SD concentration caused the more reduced cell viability.

induction of burn wounds. The test dressings were removed after 96 h (Fig. 4). Consistent with the in vitro study, co-supplementation of Ag-SD and EGF to the sponge enhanced epidermal regeneration and accelerated the wound closure, compared to the one treated with Ag-SD alone. Degrees of wound closure by drug-loaded collagen dressings are summarized in Table 2. The degrees of wound closure with EGF supplemented in collagen sponge were always higher during the course of wound healing, compared to Ag-SD alone. Also, the supplementation of Ag-SD to the sponge reduced the degree of the closure by 24% compared to the EGF treated sponge on day 7 after applying the dressing. Histological and visual observation showed that Ag-SD + EGF treatment improved the wound closure, which was apparently associated with better epithelialization which was not apparently initiated in the case of Ag-SD alone.

Histological examination was carried out in an excised skin from the burn wound at 96 h after the application of collagen sponge to the burn wound (Fig. 5). The skin samples remained moist and appeared

viable, indicating that healing process was going on in skins dressed by occlusive dressing only, by the sponge with Ag-SD and by the one with Ag-SD + EGF as shown in Fig. 5A, B, and C, respectively. Occlusive dressing alone showed a significant number of inflammatory cells (Fig. 5A, arrow). In contrast, although no significant inflammation was detected, the burn wound treated with the sponge containing Ag-SD did not show a sign of epithelialization, which may be related to an inhibitory effect of Ag-SD on epidermal cell growth (Fig. 5B). Wound treatment with the dressing containing both Ag-SD and EGF showed regeneration of the epidermis layer and no inflammatory cells (Fig. 5C).

Fig. 6 shows the required time for complete wound closure. The sponge dressing with Ag-SD required a longer time for complete wound closure which may be due to the inhibition of epidermal regeneration. This was consistent with the histological examination as shown in Fig. 5B. Interestingly, co-supplementation of Ag-SD with EGF demonstrated higher wound closure rate at EGF ($4\,\mu g/cm^2$) than at EGF ($2\,\mu g/cm^2$), indicating that the wound closure rate is dependent on EGF dosage. In contrast, the collagen dressing containing EGF without Ag-SD showed a further enhancement in wound closure rate, compared with that seen in dressing with Ag-SD and EGF.

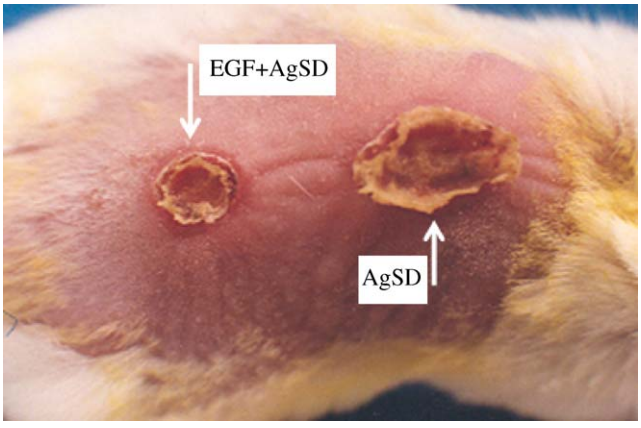

Fig. 4. Comparison of wound closure between EGF + Ag-SD and Ag-SD alone in 7 d after the induction of burn wound burn in ICR mice. Arrows indicated the wound treated with Ag-SD 2% or Ag-SD 2% + EGF ($2\,\mu g/cm^2$) in collagen sponge.

4. Discussion

Normal wound healing is a complex and sequential process involving several cell types. Fibroblasts, inflammatory cells and keratinocytes all function in an integrated manner to promote cell division, differentiation and migration. The healing of open wounds depends in part on the proliferation of keratinocytes and fibroblasts and the laying down of a collagen matrix in the dermis [15]. Ag-SD is an antibiotic that reacts with nucleic acids and thus prevent so-called unzipping of the double helix, the vital part of cell proliferation. Therefore, although Ag-SD is an antibacterial agent that is clinically useful in the treatment of wounds, the inhibition of cell proliferation by Ag-SD [3,16] may impair wound healing by directly inhibiting mitosis of fibroblasts and keratinocytes or inflammatory cells.

Since EGF is a potent mitogen for keratinocytes and fibroblasts [9], the cyto-protective effect of EGF on Ag-SD exposed cells is somewhat expected. Indeed, we have observed that co-supplementation of EGF and Ag-SD lead to an improvement in wound closure rate. However, the underlying mechanism of EGF on the higher wound-healing effect is not directly investigated in this study. In the literature, it has been postulated that Ag-SD transport into cells may be diminished in the presence of EGF and/or addition of EGF may lead to an enhanced resistance to cyto-toxic effect of Ag-SD [7].

To be able to observe the effect of EGF on the enhancement of wound-healing process, a well controlled burn model is necessary. If the burn condition is too harsh and destroys all the skin cell, regeneration of the skin will not occur. The wounded skin should contain some viable dermal fibroblasts for the production of collagen, which will lead to a dermal matrix formation (e.g., 100 °C burn condition induces third-degree burn and complete dermal destruction). In contrast, the burn damage used in this study induced partial thickness burns [17].

Table 2
Degree[a] of wound closure by drug-loaded collagen dressings in percentage

Observation time[b] (day)	EGF (4 μg/ml)	Ag-SD (1%)	Ag-SD (1%) + EGF (4 μg/ml)
7	64.6 ± 6.4[c]	40.5 ± 0.7	46.3 ± 1.2[c]
13	92.6 ± 2.0[c]	80.8 ± 1.9	92.1 ± 1.1[c]

Collagen sponges were treated with the specified solutions as described in Table 1. Freeze-dried sponges were re-hydrated with sterile 200-μl phosphate buffered saline solution before applying onto the wounds. Result represents the mean ± standard deviation from triplicate samples.

[a]Degree of wound closure = (initial wound area − wound area at specified time)/initial wound area × 100%.

[b]Time after the application of each sample onto wound model.

[c]Indicate difference with Ag-SD treated wound at the *p*-values less than 0.01.

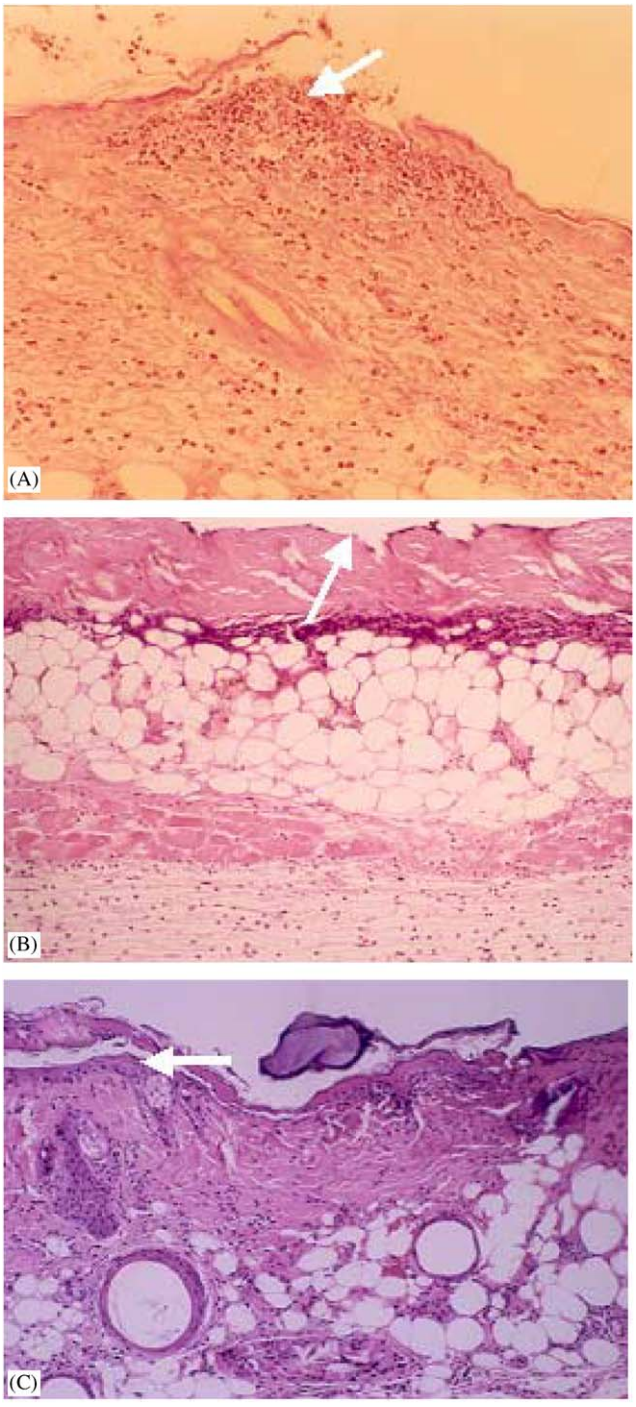

Fig. 5. Histology of H–E stained second-degree burn wound after 96 h of application of test dressings. (A) Occlusive dressing only, a significant number of inflammatory cells is present. (B) Collagen + Ag-SD (2%), due to some inhibitory effect of Ag-SD on epidermal cell growth, regeneration of epidermal cells has not progressed. (C) Collagen + Ag-SD (2%) + EGF (2 μg/cm^2), histological examination demonstrated regeneration of epidermis layer (arrow) there were no apparent inflammatory cells. For all micrographs, original magnification × 100.

Previously, EGF failed to stimulate epithelialization when applied for 5 min in saline solution. However, EGF significantly stimulated epidermal regeneration

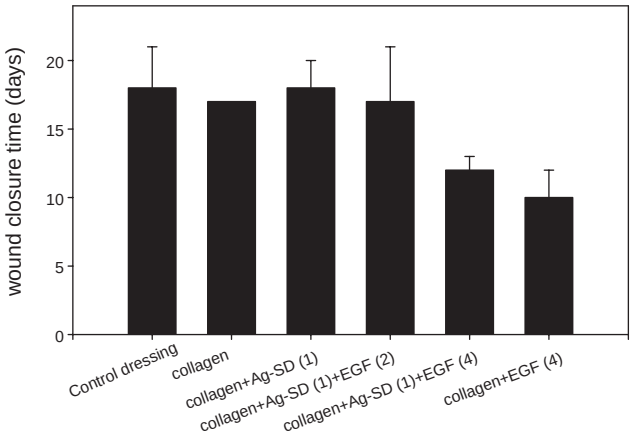

Fig. 6. Days required for complete wound closure for test dressings. Ag-SD (1%) incorporated collagen sponge dressing requires longer time to have complete wound closure due to the inhibition of epidermal regeneration. Co-supplementation of EGF shows the higher wound closure rate in EGF (4 μg/cm^2) than the one with EGF (2 μg/cm^2). Collagen dressing containing EGF without Ag-SD (1%) showed a significant enhancement in wound closure rate.

when it was applied to partial thickness incisions in creams that released EGF for prolonged periods [18]. Therefore, formulation of EGF in a simple, water-soluble vehicle that does not adequately sustain the release may not induce epidermal regeneration of the wounds. A number of literature findings pointed that an EGF formulation should release the growth factor in a continuous manner during the early phase of wound heal [19–21]. Therefore, EGF contained in a collagen sponge may be clinically useful since the formulation is likely to release EGF to the site of skin damage and is readily applicable in patients with burn wounds.

In burn wound management, fast wound closure is required to prevent secondary infection. Impaired dermal cell proliferation and uncontrolled infection may lead to scar formation. Therefore, the use of Ag-SD and EGF is necessary for the prevention of infection (i.e., by Ag-SD) and for a higher wound closure rate (i.e., by EGF). To optimize the favorable effects of Ag-SD and EGF in terms of the prevention of scar formation, identification of desired drug delivery rates is under investigation in this laboratory.

In conclusion, the inhibitory effect of Ag-SD on keratinocyte and fibroblast growth induced the retardation of wound closure rate. The addition of EGF to HaCaT cells in the presence of Ag-SD is associated with enhanced cell viability, probably by the stimulatory effect of EGF on cell proliferation. The in vivo wound-healing study showed that collagen sponges containing Ag-SD and EGF could control infection and reverse the impairment of wound healing by Ag-SD. These observations indicate that simultaneous delivery of EGF and Ag-SD may be clinically useful in the management of infection and wound closure.

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