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Enhancing Dermal Matrix Regeneration and Biomechanical Properties of 2nd Degree-Burn Wounds by EGF-Impregnated Collagen Sponge Dressing

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To better define the relationship between dermal regeneration and wound contraction and scar formation, the effects of epidermal growth factor (EGF) loaded in collagen sponge matrix on the fibroblast cell proliferation rate and the dermal mechanical strength were investigated. Collagen sponges with acid-soluble fraction of pig skin were prepared and incorporated with EGF at 0, 4, and 8 μg/1.7 cm^2. Dermal fibroblasts were cultured to 80% confluence using DMEM, treated with the samples submerged, and the cell viability was estimated using MTT assay. A deep, 2nd degree- burn of diameter 1cm was prepared on the rabbit ear and the tested dressings were applied twice during the 15-day, post burn period. The processes of re-epithelialization and dermal regeneration were investigated until the complete wound closure day and histological analysis was performed with H-E staining. EGF increased the fibroblast cell proliferation rate. The histology showed well developed, weave-like collagen bundles and fibroblasts in EGF-treated wounds while open wounds showed irregular collagen bundles and impaired fibroblast growth. The breaking strength (944.1 ± 35.6 vs. 411.5 ± 57.0 Fmax, gmm^{-2}) and skin resilience (11.3 ± 1.4 vs. 6.5 ± 0.6 mJ/mm^2) were significantly increased with EGF-treated wounds as compared with open wounds, suggesting that EGF enhanced the dermal matrix formation and improved the wound mechanical strength. In conclusion, EGF-improved dermal matrix formation is related with a lower wound contraction rate. The impaired dermal regeneration observed in the open wounds could contribute to the formation of wound contraction and scar tissue development. An extraneous supply of EGF in the collagen dressing on deep, 2nd degree-burns enhanced the dermal matrix formation.

Key words: Epidermal growth factor (EGF), Wound mechanical strength, Collagen sponge, Fibroblast, Wound healing

INTRODUCTION

With the development of genetic engineering techniques, recombinant epidermal growth factor (EGF) has become available for therapeutic uses due to its favorable effect on the enhancement of the wound healing process (Beaubien *et al.*, 1994; Franklin and Lynch, 1979; Leibowitz *et al.*, 1990). EGF enhances the fibroblast growth and collagen content of the dermis layer (Brown *et al.*, 1988; Laato *et al.*, 1986). Since the keratinocytes of the epidermis are primarily responsible for massive fluid loss and microbial invasion, initial studies focused on the replacement of keratinocytes alone. Recently, however, it has been proposed that the replacement of the dermis itself is equally important as the outer layer of the epidermis.

The mechanical properties of a healing wound depend on the physical attributes of the collagen fibers such as fiber diameter, alignment, and cross-linking, as well as on other matrix components (Laato *et al.*, 1986; Mukhopadhyay *et al.*, 2005; Piscatelli *et al.*, 1994). The complexity of wound biomechanics means that a description based on only one parameter, usually tensile strength, is often misleading (Beausang *et al.*, 1998; Kingsnorth *et al*, 1990). Thus in this study, several quantitative properties were calculated from the load versus strain curve in each tested skin specimen (Davis *et al.*, 2000) and were compared with those of normal, natural skin. The histology of the tested wound skin specimens was also analyzed with H-E staining to understand the correlation between wound

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strength and the fibroblast regeneration and collagen fiber arrangements (Cho Lee *et al.*, 2003).

Considering that dermal wound strength after partial thickness skin wounds may reflect the degree of cell proliferation of keratinocytes and fibroblasts and the alignment and density of collagen fiber networks in the dermis, this study investigated the effect of EGF by comparing the biomechanical properties of the healed EGF-treated wounds with open wound controls. As a dermal substitute, a collagen sponge matrix with EGF impregnated was prepared and its effects on the fibroblast cell growth and collagen fibril formation were investigated. Its relation with dermal wound strength was also investigated by quantifying the biomechanical parameters.

MATERIALS AND METHODS

Materials

EGF was purchased from Sigma Chemical Co. (St Louis, MO, U.S.A.) and Dulbecco's modified Eagle's medium (DMEM) and fetal bovine serum (FBS) from Invitrogen (San Diego, CA, U.S.A.). Other chemicals were of analytical grade. Human dermal fibroblast was provided by the Department of Dermatology, School of Medicine, Seoul National University. Duoderm occlusive dressing (Convatec Co., NJ, U.S.A.) was employed as a control dressing.

Collagen sponge preparation

The sponges were prepared from collagen extracted from pig skin. Briefly, 5 mL of 1% collagen solution was placed in 35 mm Petri-dishes and freeze-dried. The resulting sponge was cross-linked with di-isocyanate hexamethylene (DICH) (1%) for 10 min. Chondroitin-6-sulfate (CH_6S) solution was supplemented at the time of molding (Cho Lee *et al.*, 1999).

Effect of EGF on fibroblast proliferation and cyto-compatibility of sponge

Gamma-irradiated collagen sponges (diameter: 3 cm) were placed into six-well plastic dishes and 80,000 cells of isolated/cultured human fibroblast were seeded per 8 cm^2 collagen sponge and supplemented with 10 mL of DMEM with 10% FBS. The sponges were incubated in 5% CO_2 at 95% humidity for a week. Population and viability of the cultured cells were measured by MTT assay.

Evaluation of wound healing property

In rabbits (2.0 ± 0.2 kg), one circular wound of diameter 1 cm was made on the anterior surface of each ear. The tested dressings were applied for 96 h for the 1st dressing, followed by another 96 h for the freshly prepared 2nd dressing. After that, the treated wound area remained open until the observation time. The degree of wound exudates, sign of infection, process of re-epithelialization and wound closure rate were evaluated at predetermined time intervals until the complete wound closure day. Histology was analyzed with H-E staining. Since the wound healing process is dependent on the degree of wound exudates absorption and occlusion, a non-adherent dressing, Duoderm®, was used as a control dressing.

Quantification of biomechanical properties of the excised wounds

At 15 days post burn, the treated wound area was excised (15×25 mm^2) and quantitative biomechanical properties such as maximum tension (breaking strength, Fmax/unit area), skin toughness, resilience, percent elongation and modulus constant were determined from the load versus strain curves obtained from a constant speed tensiometer (Stable Micro Systems, UK) as reported previously (Cho Lee *et al.*, 2003).

Data analysis

At a minimum, the experiments were performed in triplicate and the average values were used for the evaluation of the biomechanical wound strength. All data were expressed as mean±standard deviation. Student's *t*-test was used to calculate the p-values to verify the difference between experimental data. Statistical significance was set at $p<0.05$.

RESULTS

Effect of EGF on the viability of cultured human dermal fibroblast

Fig. 1B and C shows the MTT assay results of the cells exposed to EGF (100 ng/mL) and 0.01% silver sulfadiazine (Ag-SD) solution, respectively. Ag-SD solution was tested as a negative control. Fibroblasts exposed to EGF exhibited cell growth stimulation while the cell viability was significantly affected by the presence of antibiotics; 0.01% Ag-SD solution as compared with control (Fig. 1A). Fig. 2 shows the fibroblast cell growth in collagen only (Fig. 2A), in EGF-loaded collagen sponge (Fig. 2B) and in 0.01% Ag-SD-loaded collagen sponge (Fig. 2C). The effects of EGF and Ag-SD on the fibroblast viability in vitro were quantified by performing MTT assay and are summarized in Fig. 3A in Petri-dish and 3-B in collagen sponge. EGF (100 ng/mL) enhanced fibroblast cell growth in both Petri-dish and collagen sponge matrix.

Comparative wound healing study in 2nd degree burn, rabbit ear, wound model

In open wounds with no dressing, contraction of the wounds without regeneration of the epidermis and dermis

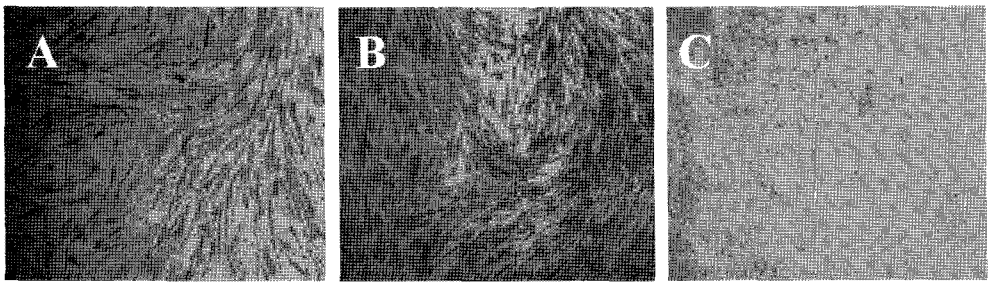

Fig. 1. Micrographs of the fibroblasts stained with MTT after 1-week culture. A: control, B: 100 μg/L of EGF, and C: 100 mg/L of Ag-SD. Ag-SD was employed as the negative control.

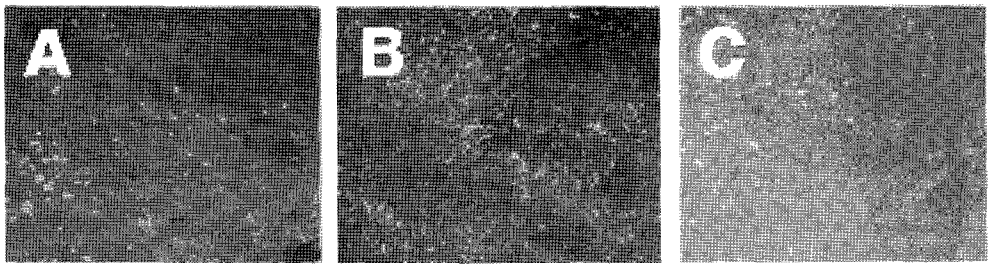

Fig. 2. Photographs of the sponges containing cultured fibroblasts for 1 week. The cells were stained with MTT. A: control, B: 100 μg/L of EGF, and C: 100 mg/L of Ag-SD. Ag-SD was employed as the negative control.

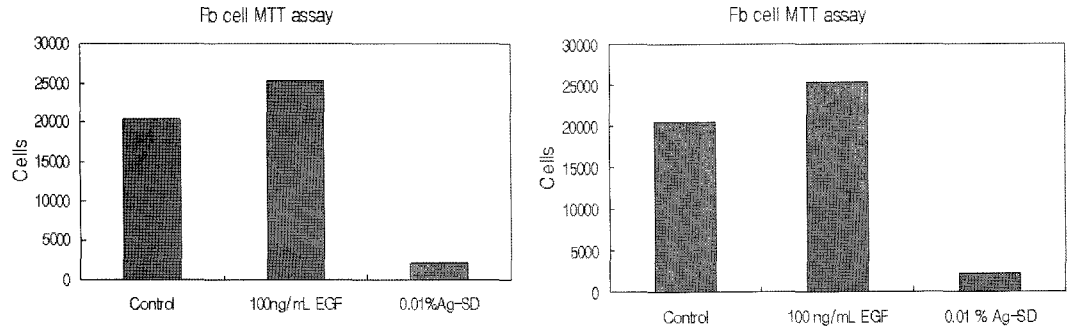

Fig. 3. Effects of EGF and antibiotics on the viability of fibroblasts in vitro were evaluated using MTT assay. (A) in Petri-dish, and (B) in collagen sponges.

layer was observed (Fig. 4A). Fig. 4B shows a wound covered with control dressing, Duoderm®, only. Partial regeneration of epidermis is observed. Collagen sponge dressing-applied wound (Fig. 4C) showed that the epidermis layer was fully regenerated except for the edge area. The wound treated with the collagen sponge containing EGF (4 μg/1.7 cm^2) showed well developed epidermis layer and full coverage of the wound (Fig. 4D).

Histological evaluation

Histological examination was carried out on excised skin from the treated wounds after the test dressing had been applied for 96 h. In open wounds with no dressing, red blood cell clotting was observed, along with many inflammatory cells without regeneration of the epidermis and dermis layer (Fig. 5A). In EGF-treated wounds, regeneration of the stratum basal layer in the epidermis was observed, along with well developed, weave-like collagen bundles and fibroblasts (Fig. 5B). Fig. 5C shows the normal skin.

Evaluation of biomechanical strength of EGF-treated wounds as compared with open wounds

Table I summarizes the biomechanical parameters of the tested wounds at 15 days post burn. In deep, 2^{nd} degree burns, the epidermis and dermis were grossly destroyed in open wounds without dressing. The EGF-treated collagen sponge increased the wound strength. The significant increase in breaking strength ($p<0.01$) and in maximum tensile strain ($p<0.02$) with the application of EGF (4 μg/1.7 cm^2) on the wound suggests that sustained release of EGF from the collagen sponge stimulates the dermal matrix formation. Considering that the physical weave of the collagen fiber is largely responsible for the

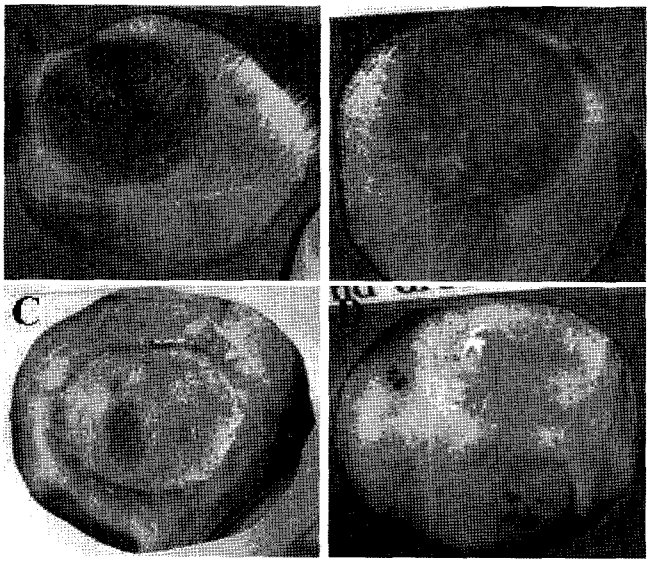

Fig. 4. Macroscopic observation of the wound healing process. A : open wound, B: Control dressing only, C: Collagen, D: collagen + EGF. Comparison of the wound healing process among open and collagen sponge wound dressing with/without EGF applied on the rabbit ear, 2nd degree-burn model. By applying EGF embedded into the collagen sponge, the process of wound repair was enhanced in terms of re-epithelialization and minimizing wound contraction.

final functional behavior of the wound (Davis *et al.*, 2000; Morine *et al.*, 1989), the increased biomechanical wound strength in EGF-treated wounds might have been due to an increased number of fibroblasts in the healing dermis, which caused it to be aligned in a more organized fashion and increased its capability of remodeling dermis due to the increased type-I collagen synthesis (Piscatelli *et al.*, 1994).

DISCUSSION

The significant loss of biomechanical properties observed in the open wounds suggests an impaired dermal regeneration with no treatment after deep, 2nd degree-burn. The stress-strain curve was split into two peaks with low elongation property and suggested the presence of irregularities of the collagen matrix distribution in open wound skin. The stress-strain curve extended over a narrow area, indicating that the work required to break the wound is small and that it has low impact resistance. Modulus (elasticity constant) is a measure of the stiffness to deformation, and the decreased value may have been due to a loss of skin elasticity.

To understand the role of collagen fibril distribution in biomechanical wound strength, we performed transmission electron microscopy (TEM) as reported previously (Cho Lee *et al.*, 2003). TEM displayed a mean diameter of 180 ± 15 nm and 148 ± 23 nm for normal and EGF-treated skin sections, respectively. However, in open wounds, the regeneration of collagen fibrils was significantly impaired

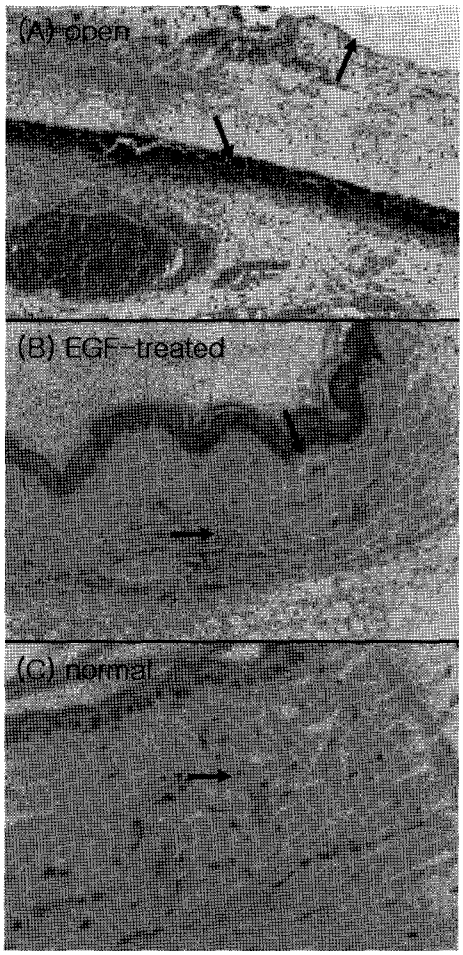

Fig. 5. Histology of H-E stained, 2nd degree-burn, wound skin sections after 96 h application of tested dressings and 7-day post burn. A: Open wound in which a lot of inflammatory cells, as denoted by the arrow, are observed. B: Collagen + EGF. Histology shows a well stained epidermis layer as denoted by the arrow and a high density of fibroblasts and collagen bundles in a weave-like pattern in the dermis layer. C: Normal, unwounded skin. Original magnification (×100).

Table I. Comparison of biomechanical properties of excised wound from 15-day, post burn, 2nd degree- burn, rabbit ear model. Data are expressed as mean±S.D. of quadruplicate measurements. Sectional area: 15 × 25 mm^2, and skin thickness of the excised wound: 1.19 ± 0.16 mm (n = 4).

Factors	EGF-treated skin	Open wound	p-value
Ultimate strength (Fmax, g/mm^2)	944.1±35.6	411.5±57.0	0.00016
Resilience (mJ/mm^2)	11.3± 1.4	6.5± 0.6	0.00528
Toughness (mJ/mm^2)	38.6± 7.1	20.5± 3.9	0.12010
Maximum tensile strain (% elongation)	35.6± 5.1	50.6±12.2	0.01789
Elasticity constant, Modulus (g/mm/mm^2)	275.6±87.5	77.3± 1.1	0.01718

and the mean diameter was less than 50 nm. EGF-treated wounds showed a well developed basket weave pattern arrangement of collagen fibrils, while open wounds showed irregular collagen bundles and impaired fibroblast growth. The impaired dermal regeneration observed in the open wounds may have contributed to the formation of wound contraction and scar formation. Considering that the fibroblasts are the major components of the dermis, impaired wound healing due to decreased fibroblasts replication may disturb the new dermal matrix regeneration and proper alignment of collagen fiber networks in the dermis, thereby decreasing the mechanical strength of the wound (Berthod *et al.*, 2001; Mustoe *et al.*, 1987).

With regard to treatment of partial-thickness burns to the skin, especially superficial dermal burns, wounds can be healed in a shorter period if the blister wall is kept intact since the wound fluid contains various kinds of cytokines which play an important role in the wound healing process (Inoue *et al.*, 1996; Eisinger *et al.*, 1998). However in severe burn conditions, such as deep, 2nd and 3rd degree burns, which have lost most of the keratinocytes and fibroblasts in the epidermis and dermis skin, an extraneous supply of growth factors is required for promoting the wound healing process (Brown *et al.*, 1988). The interaction between EGF and the cells that culminates in a mitogenic response is initiated by binding of EGF to cells at its high affinity, cell surface receptor, although initiation of DNA synthesis requires at least 8-12 hours exposure to EGF (Buckley *et al.*, 1985). With the knowledge that EGF requires prolonged, continuous exposure to stimulate cells to divide, numerous studies have stressed the importance of the delivery system (Buckley *et al.*, 1985; Celebi *et al.*, 1994). A single dose of EGF either in saline or in hyaluronic acid, which could not adequately sustain its release, failed to enhance the wound healing process (Curtsinger *et al.*, 1989). The local and sustained presence of EGF accelerates the process of wound repair, fibroblast organization, and collagen accumulation which is the major dermal matrix and is produced by fibroblasts (Buckley *et al.*, 1985; Kingsnorth *et al.*, 1990). The collagen content was increased by almost 50% and the relative rate of collagen synthesis remained the same, suggesting that the morphological and biochemical increase in collagen resulted from an increased number of fibroblasts, rather than from a specific stimulation of collagen synthesis.

However, considering the increase in ultimate tensile strength that occurs in aged skin due to the loss of elastin and the alteration of collagen alignment, increased tensile strength may not always provide favorable effects on the skin (Mukhopadhyay *et al.*, 2005; Yao *et al.*, 2001). Therefore, careful consideration should be given to gaining a balance between promoting fibroblast growth in the initial period of the healing stage and regulating the over-proliferation of fibroblasts in the later period of the dermal matrix remodeling stage. To optimize the favorable effects of EGF on the prevention of scar formation, research is presently underway to identify the desired drug delivery rate.

In conclusion, the physical weave of the collagen fiber is largely responsible for the final functional behavior of the wound. The increased dermal wound strength in EGF-treated wounds may have been due to the increased number of fibroblasts in the healing dermis, the more organized alignment, and the improved capability of remodeling due to the increased collagen synthesis. The extraneous supply of collagen and EGF on deep, second degree burns enhanced the dermal matrix formation. Improving the dermal matrix formation by EGF is related with a decreased wound contraction rate. The impaired dermal regeneration observed in open wounds may contribute to the formation of wound contraction and scarring tissue development.

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