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Original Article

Implications of the epidermal growth factor on burn skin wound repair. An *in vitro* and *in vivo* study

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Abstract: An injury healing process after tissue damage comprises of a profoundly directed succession of events. This paper outlines the role of EGF in tissue recuperating and tissue repair, and presents its clinical utility as restorative agent for different injury. The enhanced dermal regeneration by the use of EGF observed in open injuries may improve the wound healing and scar tissue. The cell viability was found to be 123%, 132% and 144% higher than those of the control group at 0.1, 0.5 and 1.0 micrometer of rLMP-EGF. The rEGF demonstrated 123%, 135% and 146% increments in cell expansion at 0.1, 0.5 and 1.0 μM, separately, thereby demonstrating that the biological activity of rLMP-EGF and rEGF was comparable. The conjugation of low molecular protamine did not adversely affect EGF activity. The rEGF treatment (10 mg/mL) reduced the injury range, when compared with the results found in the control group. An appreciable improvement was noticed on the fourth day. The injured tissue was also assessed histologically. Application of rLMP-EGF or rEGF demonstrated maximum re-epithelisation while no recovery of the epidermis was observed in injuries treated with a vehicle.

Keywords: Angiogenesis, wound healing, epidermal growth factor

Introduction

Albeit once thought to be static, wound recuperating represents a dynamic physiological process initiated and impacted by numerous contributory factors. Healing of the wounds involves the initiation of a series of sequential stages, including aggravation, wound compression, and the reorganization and redesigning of dermal tissue [1]. It includes the relocation and expansion of cells at the injury edge to recover an epithelium over the injury. In the event that the damage is limited to the epidermis, this injury-healing activity starts very quickly and may seal the injury within 24 hours. Then again, when dermal tissue is likewise harmed, epidermal cell relocation and expansion happen in parallel with wound compression and fibre network redesigning in the dermis [1-3]. Chronic wounds can be considered for growth factor treatment, since it is believed to heal the chronic lesion. Growth factor enhances healing of the wounds, by preventing hypoxia and enhancing the vascularity [4]. Vascular endothelial growth factor (VEGF) invigorates wound healing by a complex mechanism comprising of angiogenesis, collagen deposition and epithelization; it may also enhance tissue perfusion. VEGF is secreted by a number of cells during angiogenesis, particularly in the initial phases [5, 6]. Enhancement of wound healing is one of the most significant actions of VEGF. Angiogenesis resulting in wound healing includes vasodilatation, endothelial cell-migration and multiplication [7], the platelet determined development component, vascular endothelial development variable and epidermal growth factor (EGF) animate cell movement, multiplication, and new blood vessel formation-considered to be essential for effective injury recuperating and healing of the tissues. Thus, numerous development components are being utilized for treating injuries in order to cause quick injury repair and full recuperation without leaving any scar on skin [8-10]. An arginine-rich cell entering peptide (CPP) (VRSGRRGRRRRRRR) arrangement in protamine was uncovered by Yang and associates. Low molecular weight protamine (LMP) shows cell translocations intensity and can viably convey expansive particles including pro-

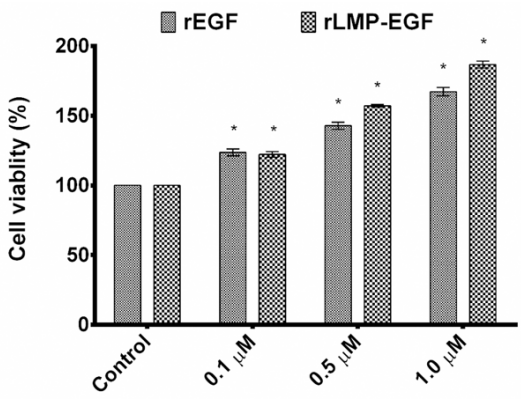

Figure 1. Growth of L929 cells incubated with different concentrations of recombinant epidermal growth factor (rEGF) or recombinant low-molecular-weight protamine conjugated EGF (rLMP-EGF) for 24 h compared to control group. Data are depicted as mean ± standard deviation, n = 2, *Significantly different from the control ($P < 0.05$).

teins [11-13]. LMP is artificially conjugated or blended through physical methods with exceedingly negatively charged macromolecules to shape a multifaceted structure with the end goal of conveying organically dynamic materials. Be that as it may, utilizing these strategies, the coupling site of LMP to macromolecules can't be regulated; therefore, the first natural movement of the macromolecule may be influenced. Moreover, different distillation steps are needed to acquire immaculate conjugate of LMP-macromolecule, which definitely lessens last yield.

This study was undertaken to observe the effects of the epidermal growth factor using the *in vitro* and *in vivo* procedures.

Materials and methods

Materials

Low molecular weight protamine (LMP), recombinant human epidermal growth factor (rEGF) and oligo arginine (R7) were supplied by Sigma-Aldrich Shanghai Trading Co Ltd (Shanghai, China). The epidermal growth factor, Biotinylated, complexed to Alexa Fluor® 555 (Alexa Fluor® 555 EGF complex) was procured from Molecular Probes, Inc. (Life Technologies, Shanghai, China). Kolliphor® P 188 (Sigma Aldrich) and used for efficacy of the polymer based ointment.

MTT cell proliferation assay

Mouse fibroblast cells (L929) were procured from the American type Culture Collection. Cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) (Invitrogen, Gibco, UK) and was added with 10% of fetal bovine serum (FBS) (Invitrogen, Gibco, UK), and antibiotics (100 IU/mL penicillin, 0.1 g/mL Fungizone) (Biological Industries, Israel) maintained at 37°C in a incubator with 5% CO_2.

The assay was performed using 96 well plates (Corning Glasswork, Corning, NY) by plating the cells at a concentration of 5×10^3 cells. Thereafter, samples were added, and both test and the control, prepared in cell culture media. Cell viability was measured by MTT cell proliferation assay [14]. All the experiments done were performed twice.

Laboratory animals

Animals were housed in cages placed adjacent to each other under environmentally controlled conditions (temperature: 22±4°C; humidity: 45-55%) with a 12 hour light/12 hour dark cycle (lights on between 08:00 and 20:00 hours). Animals were housed in cages with a maximum of four mice per cage and given food in the form of dry chow pellets and water *ad libitum*. Approval was taken from the Institutional Animal Care and Use Committee for the experimental animal procedures.

In vivo wound healing efficacy of rEGF-LMP

Using a biopsy punch, under isoflurane anaesthesia, the skin of each athymic mouse on dorsal surface was used to shape an indirect wound (8 mm in measurement). Using povidone-iodine the wound area was cleaned. Each wound area, after 1 day, was topically fortified using 30 mL of vehicle (6% (w/v) Kolliphor® P 188), recombinant epidermal growth factor in vehicle (15 mg/mL), or rLMP-epidermal growth factor (15 mg/mL) two times a day for a period of 10 days.

Estimation of wound healing efficacy

The level of wound healing on the skin was assessed by the measurement of the range of the injury utilizing an advanced camera (G9

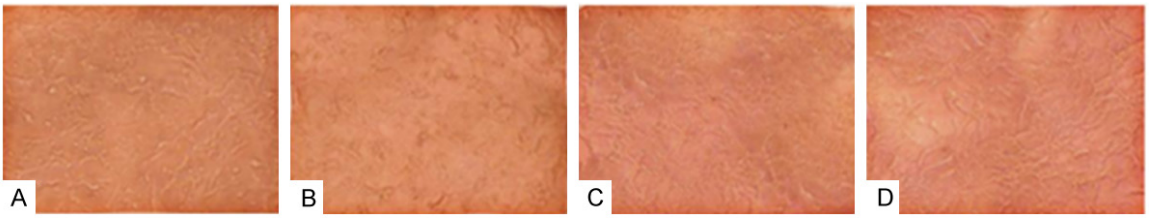

Figure 2. Illustrative microphotographs of the L929 cells in medium containing. (A) Fetal Bovine Serum only at 0 h, (B) Fetal Bovine Serum only after 24 h, (C) 0.1 μM of recombinant epidermal growth factor (rEGF) or recombinant low-molecular-weight protamine conjugated EGF (rLMP-EGF) after 24 h, and (D) 0.5 μM of rEGF or rLMP-EGF after 24 h (×100).

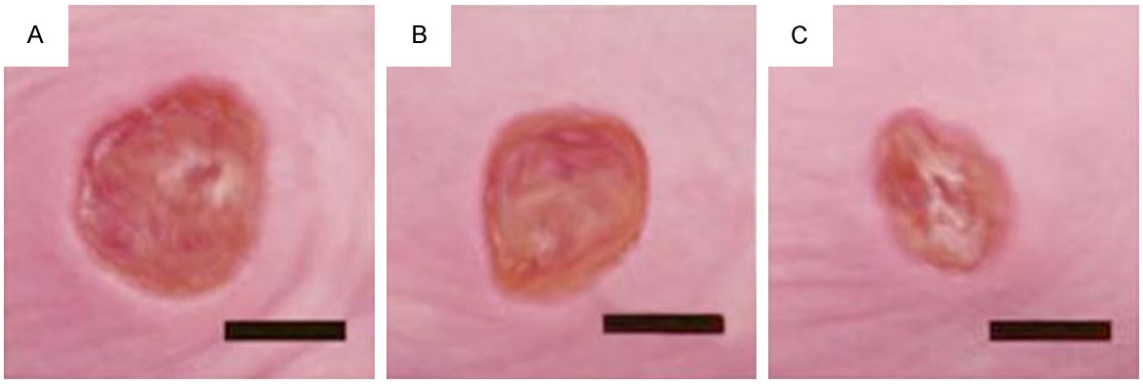

Figure 3. Shows reduction in the wound size in recombinant epidermal growth factor (rEGF) treatment group (B) recombinant low-molecular-weight protamine conjugated EGF (rLMP-EGF) treatment group when compared to vehicle treatment group (A) on day 4 (B) and 10 (C). (Bar = 500 um).

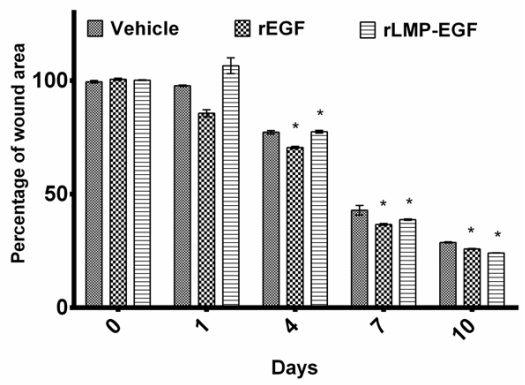

Figure 4. Outcome of epidermal growth factor (rEGF) or low-molecular-weight protamine (LMP) conjugated EGF (rLMP-EGF) on wound area reduction in the full thickness model. Percentage of wound areas on day 0, day 1, day 4, day 7 and day 10. Data are depicted as mean ± standard deviation, *significantly different from the control ($P < 0.05$).

PowerShot, Canon, Tokyo, Japan) and a picture examination software (CellSens standard, Olympus, Japan). The dimensions of remaining lesion were taken as the rate of wound healing.

Histology and immunohistochemistry

The removed injured tissue was fixed in 10% phosphate-supported formalin using standard procedure. These were processed for Paraffin embedding, with alcohol used as a dehydrating agent. Benzene was used as a clearing agent. About 4-5 μm thickness sections were cut and stained by hematoxylin and eosin staining method. A light microscope was used for the observation of inflammation and the extent of re-epithelisation. Moreover, immunohistochemical staining was done for smooth muscle actin to detect the myofibroblast separation in the lesion. The tissues, incubated with Polyclonal rabbit antibody, were used to measure the tissues against smooth muscle immune reaction as the essential immune response for 1 hour and after that with a primary antibody to rabbit HRP conjugated secondary antibody for 10 min.

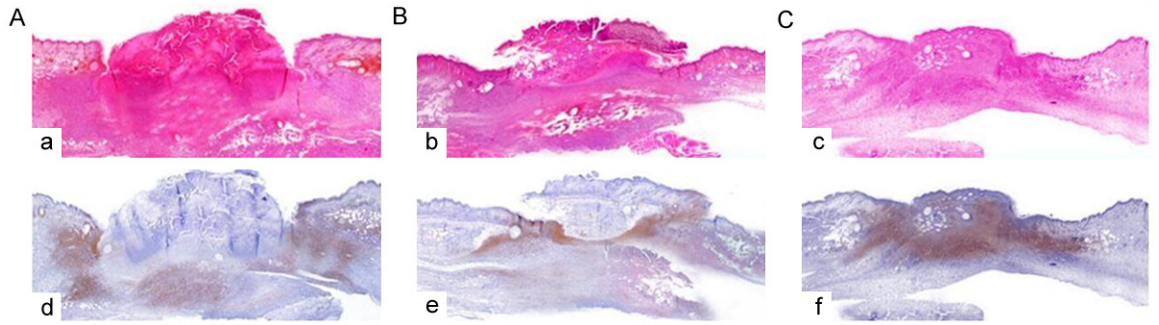

Figure 5. Effect of epidermal growth factor (rEGF) or low-molecular-weight protamine (LMP) conjugated EGF (rLMP-EGF) on wound area reduction by using H&E staining and immunoreactivities of a-smooth muscle actin (aSMA) of (A) vehicle treatment group, (B) rEGF treatment group, and (C) rEGF-LMP treatment group on day 4. Bar = 500 μm.

Statistical analysis

The percentage closure of wounds between groups at different time intervals were compared using one-way analysis of variance (ANOVA), followed by Dunnet's test. Data were analyzed using the Graph Pad Software (5.0-demo version) and P value of < 0.05 was considered to be significant.

Results

MTT assay

To assess the biological action of rLMP-EGF, the L929 cell lineage with rEGF/rLMP-EGF was inspected for cell expansion. At 0.1, 0.5 and 1.0 μM of rLMP-EGF, the cell viability values were 123%, 132% and 144% more than that of the control. Recombinant EGF demonstrated 123%, 135% and 146% increments in cell expansion at 0.1, 0.5 and 1.0 μM, separately. There was not much difference in the biological activity of LMP- EGF and epidermal growth factor. The conjugation of low molecular protamine did not adversely affect the EGF activity (**Figure 1**). The microphotographs are presented demonstrating how increasing the dose resulted in enhanced toxic effects. Concentrations greater than 0.5 and 1.0 μM resulted in an appreciable reduction in cell viability on the first day as shown in **Figure 2**.

Wound area healing

The effectiveness of rLMP-EGF *in vivo* was measured on the mice wound model. Areas on rEGF application (10 mg/mL) diminished the damaged regions, which was easily differentiated from that in control areas. Appreciable difference was noticed on day 4. Areas of the wound on rLMP-EGF demonstrated significant healing. A significant degree of healing was observed on days 4 and 10. The lesion size on days 4 and 10 were 68±3.1 and 24±3.1 (**Figure 3**). The **Figure 4** demonstrates the effects of the rEGF on the wounds and its healing extensions. The injury closure was contrasted with the control wound. Moreover, injured tissue was examined histologically. Under the treatment of rLMP-EGF or rEGF, re-epithelisation was fundamentally significant. On the other hand, no recovery of the epidermis could be noticed in vehicle-treated-injury. Nearby those lesions, there was lesser provocative and proliferative response in rLMP-EGF and rEGF-treated injuries which could be easily differentiated from the wounds treated with just vehicle, demonstrating that rLMP-EGF and rEGF-treated lesions had entered proliferative stage (**Figure 5**).

Discussion

A lot of research is going on the exogenously developed agents that may magnify cell expansion and enhance the wound repair. These agents may have an impact on epidermal cell mitosis by affecting the activity of epidermal development component and transforming growth. These particles are found in membrane-bound and soluble form, and show their activity through juxtacrine and paracrine route [15-17]. During the past few years, a series of key candidate players in the wound-healing scenario have been identified. One of VEGF's role in wound healing is incitement of angiogenesis. The fibroblast and keratinocyte multiplication around injuries happens in the initial phas-

es of healing. The role of EGF is very fundamental at the beginning phase of wound healing, in light of the fact that EGF enhances fibroblast and keratinocyte expansion within one day after expansion. It also increases the levels of epiregulin, keratin and loricrin [18-22]. Topically or subcutaneously administered EGF quickens reepithelialisation and expands rigidity of the injury. Consequently, EGF has been noted for treating impeded dermal injuries and more importantly, the extent of its application is presently extended to treatment of chronic injuries, for example, smoulder wounds and incessant ulcers. Nonetheless, there are a few hindrances to expanding the injury-recuperating adequacy of EGF. There are several complications associated with enhancing the EGF activity in the epidermal and dermis zones around the wound, including enhanced loss through skin and diminished development. Several advanced drug transport modalities, for example, EGF immobilization or encapsulation/embodiment in a polymer system and nuclear regulation of EGF have been performed to enhance EGF activity by interaction with oppositely charged glycans on the cell surface [21, 23-26].

Conclusion

Cutaneous injury healing is an interesting procedure consisting of various phases including leukocyte enrolment, framework strengthening, epithelialization and eventually replacement by a full grown scar. Bleakness connected with age-related deferred injury mending causes a tremendous social and economic cost; unless enhanced injury care systems are created, the anticipated increase in the elderly population will further worsen this issue. Prolonged healing is associated with faulty inflammatory response and lattice degeneration, thereby signifying that *in vivo* wound healing is a complex process involving disruption of skin discontinuity. Future remedial procedures should be focussed upon particular cell processes, for illustrating the nullification or upregulation of hormonally controlled qualities or particular proteins items, all working together to quicken mending in experimental animal creatures, and eventually human models.

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Disclosure of conflict of interest

None.

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