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# Recombinant human granulocyte macrophage colony-stimulating factor (rhGM-CSF) could accelerate burn wound healing in hamster skin

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Burns are one of the most devastating forms of trauma and wound healing is a complex and multicellular process, which is executed and regulated by signaling networks involving numerous growth factors, cytokines, and chemokines. Recombinant human granulocyte macrophage colony-stimulating factor (rhGM-CSF) was specifically produced from rice cell culture through use of a recombinant technique in our laboratory. The effect of rhGM-CSF on promotion of deep second-degree burn wound healing on the back skin of a hamster model was evaluated through a randomized and double-blind trial. As macroscopic results, hamster skins of the experimental groups showed earlier recovery by new epidermis than the control groups. Immunohistochemical reactions of proliferating cell nuclear antigen and transforming growth factor-β1, which are indicators of cell proliferation, were more active in the experimental group, compared with the control group. On electron microscopy, basal cells in the epidermis of the experimental group showed oval nuclei, prominent nucleoli, numerous mitochondria and abundant free ribosomes. In addition, fibroblasts contained well-developed rough endoplasmic reticulum with dilated cisternae. Bundles of collagen fibrils filled the extracellular spaces. Particularly, ultrastructural features indicating active metabolism for regeneration of injured skin at 15 days after burn injury, including abundant euchromatin, plentiful free ribosomes, and numerous mitochondria, were observed. These findings suggest that use of rhGM-CSF could result in accelerated deep second-degree burn wound healing in animal models.

**Keywords:** burn; hamster skin; rhGM-CSF; wound healing



## Introduction

Burns are common and devastating forms of trauma because loss of the functional skin barrier leads to increased susceptibility to infection, the major cause of morbidity and mortality following burns. Burns are classified according to depth of burn, and may be first-, second-, or third-degree, sometimes known as superficial, partial thickness, or full-thickness, respectively. A deep partial skin thickness burn is an excellent wound model for use in study of the main components of burn wound healing processes (Jackson 1953; Khodadadi et al. 2008).

Wound healing is a complex and dynamic biological process. In an attempt to improve wound healing, various agents, including granulocyte macrophage colony-stimulating factor (GM-CSF), basic fibroblast growth factor, and GM-CSF hydrogel, have been used. Although GM-CSF has been applied successfully in treatment of various clinical problems, its application in burn treatment has not been well reported (Dorsett-Martin 2004; Méry et al. 2004; Cianfaran et al. 2006).

GM-CSF specifically stimulates bone marrow progenitor cells destined to become granulocytes and macrophages *in vivo* and *in vitro*. Secretion of GM-CSF by keratinocytes, which occurs in skin shortly after injury, mediates epidermal cell proliferation in an autocrine manner. Thus, GM-CSF is a pleitropic cytokine, evoking complex processes during wound repair (Braunstein et al. 1994; Masucci 1996; Fang et al. 2007; Barrientos et al. 2008).

The human GM-CSF gene is located in the long arm of chromosome 5 at positions q23–q31, and contains four exons and three introns, which span an area of approximately 2.5 kb. In the mouse, the GM-CSF gene is located on chromosome 11, at bands A5 to B1. Under normal conditions, GM-CSF can be detected in serum at concentrations ranging from 20 to 100 pg/mL.

Both human and murine GM-CSFs have been identified as glycoproteins that displayed similar molecular weight (24–33 kDa). Human and murine GM-CSF proteins only share 52% amino acid identity and do not cross-react in their biological activities. Other than human and mouse, sequences encoding for GM-CSF from a variety of species have been identified (Gasson 1991; Barreda et al. 2004; Arellano and Lonial 2008). Meanwhile, recombinant human GM-CSF (rhGM-CSF), which contains more oligosaccharide

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side chains than any other types of GM-CSF, was specifically produced from rice cell culture through use of a recombinant technique in our laboratory (Kim et al. 2008).

In this study, we evaluated the efficacy of rhGM-CSF for promotion of deep second-degree burn wound healing through a randomized, double-blind trial. Morphological and immunohistochemical examination of hamster skin during the burn wound healing process stimulated by application of rhGM-CSF was conducted.

## Materials and methods

### *Animals*

Adult male golden hamsters were housed individually in order to prevent traumatic damage to wounds by other hamsters post-injury, with free access to food and water, and were kept in a constant environment ($20\pm2^\circ$C, $50\pm10$% humidity, 12 h light/dark cycle). Where appropriate, hamsters were anaesthetized with ethyl ether and pain was relieved with analgesics administered by intraperitoneal injection.

### *Burn injury*

Backs of hamsters were shaved and swabbed with 70% alcohol. A cylindrical template (1.5 cm in diameter) was heated electrically prior to injury at a constant temperature of 90°C. In order to achieve a significant reduction of the biological variables among the various treated groups of burns in a set of experiments, we inflicted two mirror-image wounds on the back of each animal, so that each treated burn had its own control on the contralateral side of the animal. Under light anesthesia, the heated template was applied at a right angle to the skin of the animal's back for 10 sec using an analogue stopwatch. Three to five animals were analyzed each time and experiments were repeated twice.

### *Treatment*

rhGM-CSF was supplied by CHA Bio & Diostech Ltd., Co. (Seoul, Republic of Korea). The rhGM-CSF, which contains more oligosaccharide side chains than any other types of GM-CSF, was specifically produced from rice cell culture through a recombinant technique. The concentration of rhGM-CSF in ointment was 50 μg/ml of base cream; 100 μL of ointment was applied on each wound every day.

### *Wound analysis*

For comparison with the original wound size, skin wound sites in each animal were digitally photographed at the indicated time intervals, and wound closure was determined using Image-Pro Plus under double-blind conditions.

### *Light microscopy*

Skin tissues were removed, cut into slices, and fixed with 10% neutral buffered formalin (v/v) for two days at 4°C. Tissue slices were dehydrated in graded ethanol and embedded in paraplast, and were cut then into slices of 4 μm thickness. Tissue sections were deparaffinized and rehydrated in graded ethanol, followed by staining with hematoxylin and eosin, and examined by light microscope.

### *Immunohistochemistry*

Paraffin embedded sections were hydrated and washed with phosphate buffered saline (PBS). All steps were carried out at room temperature in a humidified chamber. To block endogenous peroxidase activity, sections were treated with 1% hydrogen peroxide diluted in PBS for 10 min. Non-specific reaction was blocked by incubating the sections for 1 hr with normal goat serum diluted 1:1 in PBS-10% bovine serum albumin. Sections were incubated with primary antibodies (anti-PCNA and anti-transforming growth factor-β1[TGF-β1]) overnight at 4°C and were washed three times with PBS. Samples were treated with biotinylated secondary antibody and incubated in avidin plus biotinylated horseradish peroxidase enzyme for 30 min, and washed in PBS. Sections were then stained with diaminobenzidine. Where necessary, samples were counterstained with hematoxylin and examined by light microscope.

### *Electron microscopy*

For observation of ultrastructural changes of various cells in skin, tissues were cut into pieces measuring approximately 1 $mm^3$ and pre-fixed in 4% glutaraldehyde in 0.1 M phosphate buffer (pH 7.2) at room temperature for 2 hr. After rinsing in phosphate buffer, the samples were post-fixed in 1% osmium tetroxide ($O_SO_4$) in phosphate buffer at room temperature for 1 hr. After rinsing in phosphate buffer, the samples were partially dehydrated with ethanol and embedded in epon mixture. Thin sections were cut on an ultramicrotome (Leica Co.) and collected on a copper grid. They were then stained with uranyl acetate and lead citrate, and

observed with a JEM-1010 transmission electron microscope (JEOL Co.).

## Results

### *Wound analysis*

Macroscopically, wound size after three days of rhGM-CSF application was unexpectedly larger than that observed immediately after burn induction. At six days, we observed scabs to be shedded from skin in the control and experimental (rhGM-CSF treated) groups. Wound size of the experimental group showed a reduction after nine days of burn injury, compared with that of the control group. At 15 days post-injury, skin showed almost complete recovery by new epidermis in the experimental groups. That is, the wound was completely re-epithelialized. In measurement of wound sizes, closure showed more rapid progress in the experimental group, compared with the experimental group, particularly between six days and twelve days (Figure 1). In addition, no sign of inflammation was observed in the experimental groups during the entire experimental period.

### *Light microscopy*

Evaluation of wounds on various days after wound injury showed a close correlation between the macroscopic and microscopic appearances of wounds. Epidermal cells were not observed in the control and experimental groups at three days, and then the dermis was first regenerated; thereafter, repair of epidermis occurred through migration and proliferation of keratinocytes. By six days, neo-epidermis that was thicker than normal epidermis was observed in both groups. Vascularizations were found more frequently in dermis of the experimental group than in the control group. In many cases, new epithelium had completely covered the wound in the experimental group at 15 days. Briefly, cell proliferation and re-epithelialization in the skin after burn injury were more active in the experimental group than in the control group (Figure 2).

### *Immunohistochemistry*

Immunohistochemically, positive reactions of PCNA and TGF-β1, which are representative indicators of cell proliferation in tissue, showed similar patterns in most experimental groups. In the early period after burn injury, more immunoreactive cells were observed in the experimental group. Relative densities of positive reaction for those proteins were comparatively higher in the experimental group than in the control group. That is, rhGM-CSF treatment resulted in a significant increase in the number of PCNA-positive cells in the epidermis of the neo-epidermis, compared with the control group; in particular, PCNA reactivity was strongest at nine days in the experimental group (Figure 3). In addition, TGF-β1 expression in the experimental group seemed to be high compared with the control group, and more immunoreactive cells for TGF-β1 were observed in the experimental group throughout the whole period (Figure 4).

### *Electron microscopy*

Ultrastructurally, basal cells in the epidermis of the experimental group at nine days post-injury contained oval nuclei, prominent nucleoli, numerous mitochondria and abundant free ribosomes. Fibroblasts contained well-developed RER with dilated cisternae. Bundles of collagen fibrils filled the extracellular spaces. Meanwhile, some signals of inflammatory activity, as evidenced by the presence of some neutrophils, were observed in the control group, (Figure 5). At 15 days post-injury, interdigitations of cytoplasmic among spinous cells and keratinized stratum corneum were often observed in the control group. In the case of

Figure 1. Relative wound sizes in the control and experimental groups on various days after burn injury.

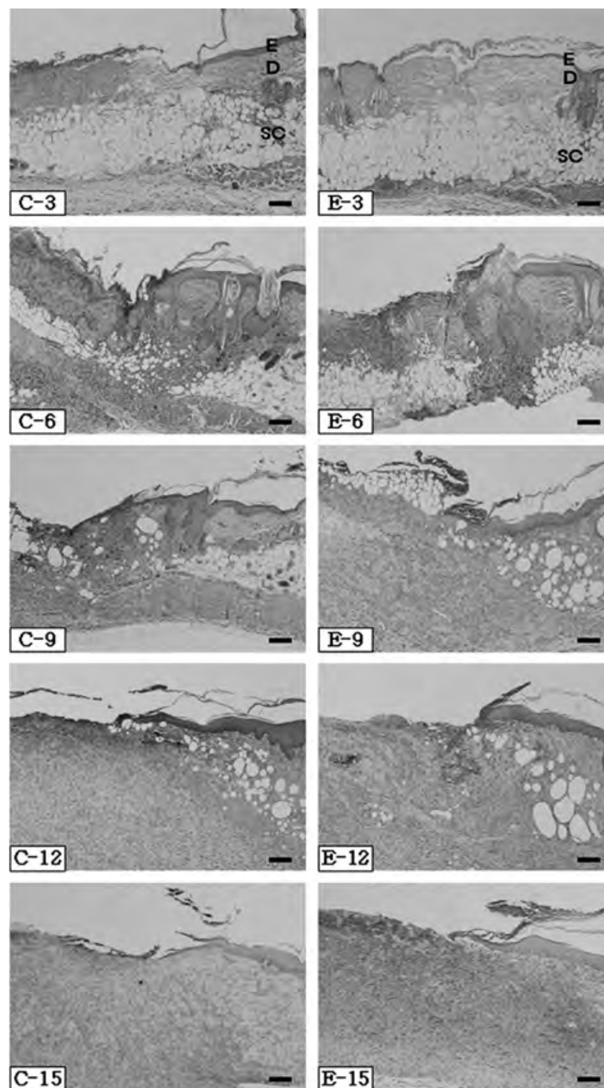


Figure 2. Light micrographs showing the histological features during wound healing on various days after burn injury. Insert on the lower left of each picture represents the control group as C and the experimental group as E, and the days after burn injury. D: dermis, E: epidermis, SC: subcutaneous layer. Scale bar: 200 μm).

the experimental group, ultrastructural features, including abundant euchromatin, plentiful free ribosomes, and numerous mitochondria, seemed to reflect active metabolism for regeneration of injured skin in (Figure 6).

## Discussion

GM-CSF is a cytokine with pleitropic functions. Found in lung-conditioned medium, it was named for its ability to stimulate formation of granulocyte and macrophage colonies. With progress in basic and clinical research over the last 10 or more years, application of rhGM-CSF in treatment of burns has become increasingly popular. Clinicians have always paid greater attention to treatment of deep second-degree burn wounds (Hill et al. 1995).

Activity and quantity of rhGM-CSF play important roles in the compromised immune function of burn animals. Topical application of rhGM-CSF on burn wounds starts a cascade of reactions for the induction of trauma repair for improvement of wound healing. RhGM-CSF promotes proliferation and activation of mature macrophages, neutrophiles, endothelial cells, epithelial cells, and fibroblast cells, which are important in the reparative process after trauma. Underlying mechanisms of rhGM-CSF include attracting inflammatory cells and endothelial cells with

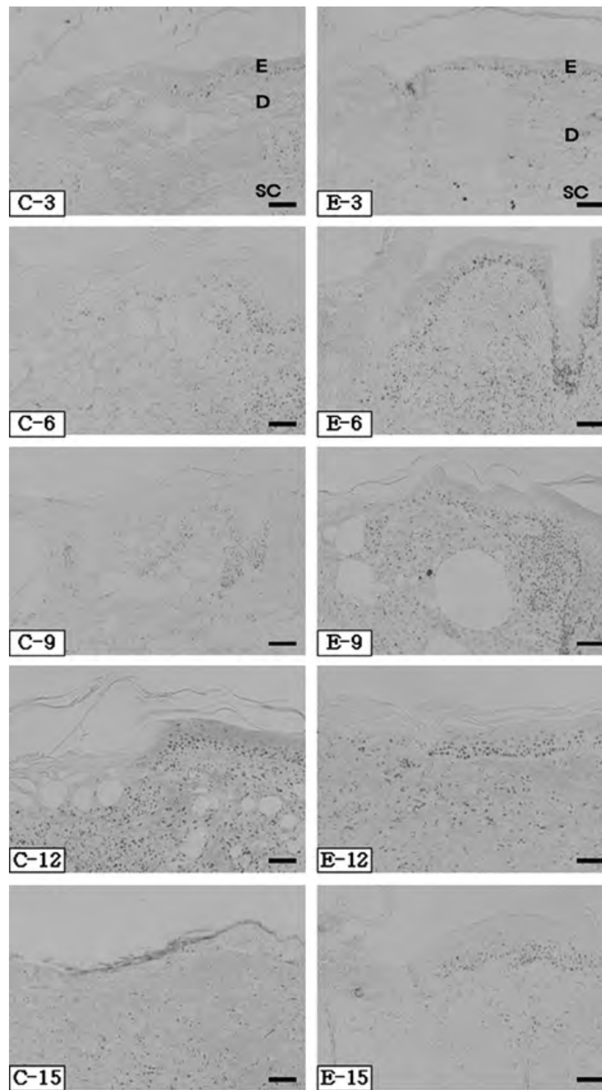


Figure 3. Immunohistochemistry for proliferating cell nuclear antigen showing the relative positive reactions during wound healing on various days after burn injury. Insert on the lower left of each picture represents the control group as C and the experimental group as E, and the days after burn injury. D: dermis, E: epidermis, SC: subcutaneous layer. Scale bar: 100 μm).

subsequent prevention from escaping the wound, with proliferation and differentiation of epidermal keratinocytes, and attraction of Langerhan's cells into dermal layers (Gasson 1991; Braunstein et al. 1994; Masucci 1996; Matsumotoa and Nakamurab 1999).

In the present study, we evaluated the efficacy of rhGM-CSF in promotion of deep second-degree burn wound healing with respect to morphology and immunohistochemistry. As a result, our study showed that topical application of an rhGM-CSF ointment to deep second-degree burns can result in significantly higher effectiveness, compared with the control group.

We observed that differences in wound size were more remarkable in ninth day wounds, that is, wound sizes of the rhGM-CSF treated group (experimental group) were significantly reduced after nine days of burn injury, compared with those of the control group.

Fifteen days post-injury, skin had recovered in the experimental groups, indicating complete re-epithelialization. This may be due to cell migration and proliferation of neighboring cells after burn injury.

Light microscopy showed early regeneration of dermis; repair of epidermis then occurred through migration and proliferation of keratinocytes. Cell proliferation and re-epithelialization in skin after

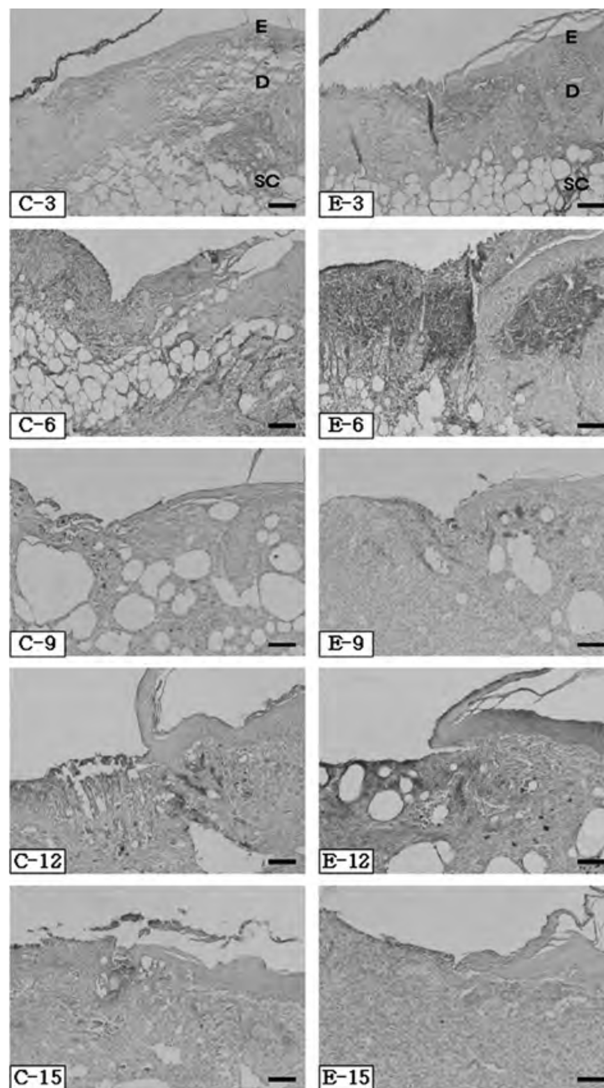


Figure 4. Immunohistochemistry for transforming growth factor-1 showing the relative positive reactions during wound healing on various days after burn injury. Insert on the lower left of each picture represents the control group as C and the experimental group as E, and the days after burn injury. D: dermis, E: epidermis, SC: subcutaneous layer. Scale bar: 100 μm).

burn injury were more active in the experimental group than in the control group. This observation suggests the important role of rhGM-CSF in wound healing compared with other types of GM-CSF (Fang et al. 2007; Barrientos et al. 2008).Many factors are involved in repair of skin wounds. Like other researchers, we have been particularly concerned with PCNA and TGF-1. Immunohistochemically, positive reactions of PCNA and TGF-1, which are representative indicators of cell proliferation in tissue, showed similar patterns. In the early period after burn injury, more immunoreactive cells were observed in the experimental group than in the control group. Relative densities of positive reaction for those proteins were higher in the experimental group than in the control group. Researchers have suggested that rhGM-CSF induces other growth factors or cytokines in a paracrine manner and that these factors stimulate keratinocytes and fibroblasts (Yamamoto et al. 2007; Haroon et al. 1999).

On electron microscopy, basal cells in the epidermis of the experimental group showed oval nuclei and prominent nucleoli, and abundant free ribosomes, indicating active protein synthesis. And fibroblasts contained well-developed RER with dilated cisternae. As a result, bundles of collagen fibrils filled the extracellular spaces. In some cases in the control group, signals of inflammatory activity, as evidenced by the

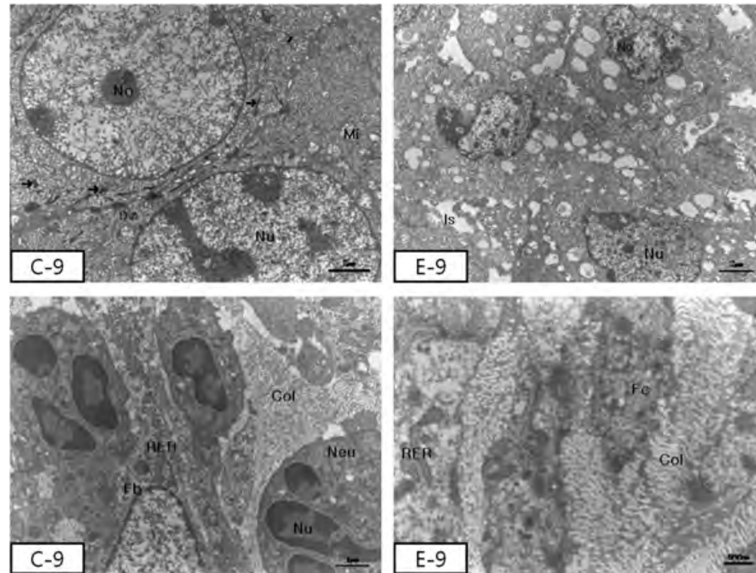


Figure 5. Electron micrographs of hamster skin of the control (left) and experimental (right) groups at 9 days after burn injury. Note the intense inflammatory activity indicated by polymorphonuclear infiltration in the control group. The extracellular matrix is composed of unorganized collagen fibrils in the control group. Insert on the lower left of each picture represents the control group as C and the experimental group as E, and the days after burn injury. Col: collagen, D: desmosome, Fb: fibroblast, Fc: fibrocyte, Is: intercellular space, Mi: mitochondrion, Neu: neutrophil, No: nucleolus, Nu: nucleus, RER: rough endoplasmic reticulum, arrows: tonofibrils.

presence of some neutrophils, were observed. Fifteen days after burn injury, ultrastructural features, including abundant euchromatin, plentiful free ribosomes, and numerous mitochondria, appeared to reflect active metabolism and proliferation for regeneration of injured skin (Odland and Ross 1968).

No adverse reactions related to the test drug were observed. From the above results, we conclude that

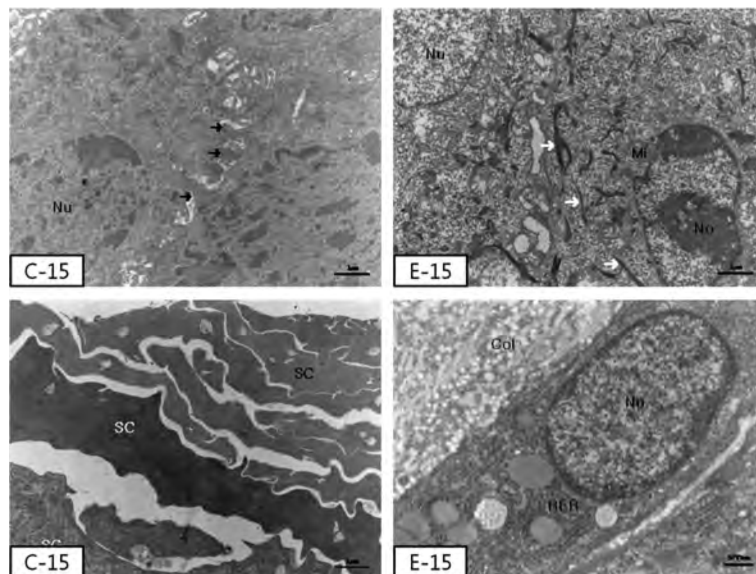


Figure 6. Electron micrographs of hamster skin of the control (left) and experimental (right) groups at 15 days after burn injury showing fibroblasts with abundant and well-developed rough endoplasmic reticulum with dilated cisternae in the experimental group. Bundles of collagen fibrils fill the extracellular spaces in the experimental group. Insert on the lower left of each picture represents the control group as C and the experimental group as E, and the days after burn injury. Col: collagen, Mi: mitochondrion, No: nucleolus, Nu: nucleus, RER: rough endoplasmic reticulum, SC: stratum corneum, SG: stratum granulosum, arrows: interdigitations of cytoplasmic processes, white arrows: tonofibrils.

rhGM-CSF is safe and effective in promotion of wound healing of deep second-degree burns. In conclusion, since rhGM-CSF derived from cultured rice cells was highly glycosylated, its effectiveness and stability could be retained longer during burn wound healing in the hamster model. Results suggest that rhGM-CSF might be useful in further development of therapeutic manipulation of burn wound healing.

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