

WOUND HEALING/EXPERIMENTAL

Granulocyte-Macrophage Colony Stimulating Factor and Steroid on Neovascularization and Keratinocyte Proliferation in Wound Healing

Zainul Naim, Laureen Supit, Eddy Sutrisno, Frank Bietra Buchari
Medan, Indonesia

Background: This study aim to evaluate the effect of subcutaneous application of recombinant human granulocyte-macrophage colony stimulating factor (rhGM-CSF) around wounds and how it influences the speed of wound healing.

Methods: The study utilizes *Mus musculus* mice in a controlled laboratory setting. Mice are divided into 3 groups: A (n = 4) receiving rhGM-CSF 10µg/kg, B (n = 4) receiving dexamethasone 10 mg/kg and C (n = 5) receiving placebo as control. Full thickness wound was made, and either rhGM-CSF, dexamethasone, or nothing were given on the wound subcutaneously for 6 days. On day 7, all rats were sacrificed and a 4-mm area from the edge of the wound were subjected for histologic examinations. Pattern of neovascularization and keratinocyte proliferation were analyzed.

Results: The data shows a higher rate of neovascularization and keratinocyte proliferation in the rhGM-CSF group compared to the steroid and placebo groups (p = 0.001). Not difference in the rate of keratinocyte proliferations (p = 0.085) and neovascularization (p = 0.935) are found between the dexamethasone and control groups.

Discussion: Granulocyte macrophage colony stimulating factor (GM-CSF) hastens wound healing in wildtype mice by increasing the rate of keratinocyte proliferation and neovascular formation, while dexamethasone has a tendency to hinder wound healing because it acts as a GM-CSF inhibitor.

Keywords: GM-CSF, Dexamethasone, Keratinocytes, Neovascularization

Latar belakang : Tujuan dari penelitian ini adalah untuk melihat pengaruh pemberian recombinant human granulocyte-macrophage colony stimulating factor (rhGM-CSF) perilesi pada luka yang dibuat guna mengakselerasi kecepatan penyembuhan luka.

Metodologi: Studi ini menggunakan mencit *Mus musculus*, dilakukan di laboratorium hewan terkontrol. Luka full-thickness dibuat pada mencit yang dibagi dalam 3 kelompok: A (n=4) menerima rhGM-CSF 10µg/kg, B (n=4) menerima dexamethason 10 mg/kg, dan C (n=5) tidak menerima apa-apa sebagai kontrol. rhGM-CSF maupun dexamethason diberikan disekitar luka secara subkutan selama 6 hari, dan pada hari ke 7 kesemua hewan coba dieuthanasia. Luka dieksisi pada batas 4 mm dari tepi luka kemudian dilakukan pemeriksaan histopatologis untuk perhitungan pertumbuhan keratinosit dan neovaskularisasi.

Hasil : Data yang didapat menunjukkan hasil pengaruh positif pemberian rhGM-CSF peri lesi subkutan terhadap pertumbuhan keratinosit dan neovaskular (p=0.001) dibanding dexamethason dan kontrol. Tidak didapat perbedaan signifikan antara kelompok dexamethason dan kontrol dalam pertumbuhan keratinosit (p=0,085) serta pertumbuhan neovaskular (p=0,935). Disimpulkan bahwa pertumbuhan keratinosit dan neovaskularisasi pasca penyuntikan rhGM-CSF lebih terstimulasi dibanding pada grup dexamethason dan kontrol, sementara tidak dijumpai perbedaan pertumbuhan keratinosit dan neovaskularisasi antara kelompok dexamethason dan kontrol.

Kata kunci : GM-CSF, Dexametason, Keratinosit, Neovaskular

Wound healing is a complex dynamic process in response to a deliberate or accidental trauma, which aims to return the local homeostatic balance in order for anatomical and functional repair to take place¹⁻⁴. Delayed wound healing is commonly faced in everyday clinical settings and carry

great morbidity as well as psycho socio economic burden for the patients and their caregivers. The synchronized process of wound healing consist of an array of programmed coagulation, inflammation, cell proliferation and migration, angiogenesis, matrix formation, remodeling, and secondary contraction^{2, 5-7}.

From Division of Plastic Surgery, Department Of Surgery, Adam Malik General Hospital, Universitas Sumatera Utara.

Disclosure: The authors have no financial interest to declare or commercial affiliations in relation to the content of this article.

Attempts have been made to study and provide the ideal wound environment and supporting factors to facilitate healing without unnecessary delay or complications. Various cellular components play role in response to injury during the four phases of healing: coagulation, inflammation, proliferation, and remodeling. Failure or disturbance during one of these phase will delay the healing process^{1, 2, 8, 9}.

This study highlight the role of a specific cytokine, the granulocyte-macrophage colony stimulating factor (GM-CSF), a multi-potent growth factor which plays an integral part in the process of wound healing for inflammatory response, re-epithelialization, and neovascularization. GM-CSF is a polypeptide with a molecular weight of 20 kDa. It is an important regulator of the proliferation, maturation, and activation of neutrophilic granulocytes. Produced by various cells including the monocytes, vascular endothelial cells, fibroblasts, and mesothelial cells, the normal circulating GM-CSF level in a healthy adult is approximately 30 pg/mL. Under biologicaly stressful condition such as during an active systemic infection, GM-CSF in blood can be elevated up to 2000 pg/mL. Human recombinant of GM-CSF (rhGM-CSF) are frequently used in the clinical settings to enhance the leucocyte concentration post-chemotherapy or to mobilize progenitor cells after bone marrow transplantation^{10, 11}.

In the process of wound healing, the keratinocytes in the basal epidermal layer secrete GM-CSF and this will accelerate wound re-epithelialization. Within a few hours after an epidermal layer is wounded, the mRNA of GM-CSF are found accumulated within the keratinocytes. It is the first responder in the body's attempt of wound healing and tissue remodeling. In vitro studies demonstrated that even in nanogram per mililiter concentration, GM-CSF acts as a potent mitogenic substance which directly stimulates the migration and proliferation of endothelial cells as well as keratinocyte proliferation in humans⁸. It is said that GM-CSF affect the proliferation, maturation, and cells recruitments by modulating cytokines release including the interleukin-1, interleukin-6, tumor necrosis factor α , tumor growth factor β , interferon γ , and macrophage

colony stimulating factors; all of which plays important role in the process of wound healing.

GM-CSF has been shown to hasten the healing of chronic ulcers¹². Intradermal application of GM-CSF to skin lesions of patients with leprosy and showed an accelerated healing of the lesions as well as an increased number of keratinocyte layers¹³. A favorable effect of GM-CSF are seen when applied to wounds inflicted on transgenic mice^{1, 2, 8, 9, 13}, especially in enhancing the rate of keratinocyte proliferation. Mice with over-expression of GM-CSF were also found to have an enhanced neovascularization and faster rate of wound healing⁸. In contrast, laboratory animal models in excess of GM-CSF antagonist have reduced rate of microvascular genesis and higher failure of wound healing, hence a direct correlation between GM-CSF to wound angiogenesis can be established. One study shows that GM-CSF supplementation for wounds with normal healing cascade offers no additional benefit¹⁴.

It has been long established that glucocorticoids exerts one of the most effective anti inflammatory effects. Because GM-CSF is cytokine, a proinflammatory substance, its release can and will be inhibited by glucocorticoids as an antiinflammatory agent¹⁵. Study on topical application of steroid on wounds is shown to hamper the effects of GM-CSF¹⁶. Experiments on mice by Mann in 2006 found a decreased mitotic activity in the basal layer of interfollicular epidermal cells, lower rate of angiogenesis, and an increased fibrotic activity after wound is exposed to a GM-CSF antagonist². Several glucocorticoids act as GM-CSF inhibitors such as fluticasone propionate, butixicort, budesonide, and dexamethasone. Fluticasone propionate and budesonide are about five-fold more potent GM-CSF inhibitors than dexamethasone¹⁵.

This study aims to investigate the effect of GM-CSF and GM-CSF antagonist application in wounds, how they influence the rates of keratinocyte proliferations and neovascularization. With this shall come a better understanding of the role of GM-CSF in the physiology of wound healing process.

MATERIALS AND METHODS

The experimental animal-model study utilized the wild type *Mus musculus* mice, with ethical clearance obtained from the Health Research Ethical Committee of North Sumatera, Medical School Universitas Sumatera Utara. Research is conducted in an animal laboratory of the above-mentioned university on December 2009 to January 2010. Fifteen healthy male mice aged between 5 to 6 months with body weight between 40-60 g were equally divided into three groups: the granulocyte-macrophage colony stimulating factor group (GM-CSF), the steroid group (STR), or the control group (CTR). Mice weighing less than 40 g and/or those with skin lesions were excluded. Subject is dropped out if death occurs within five days after intervention.

Prior to any procedure, fifteen mice were separated from their litters and placed individually into 30 by 30 by 15 cm plastic transparent boxes. They were left untouched within the boxes to adapt for seven days in a room temperature with indirect sunlight exposure, and fed ad lib. Mice were weighed on the day of surgery, then anesthetized by trans-peritoneal injection of 0.05 mg Ketamine per 20 mg body-weight, and placed prone with four limbs taped onto an operating field. Hair was removed from some area on the paravertebral region manually by razor, and skin disinfected using 10% Iodine solution then 70% Ethanol. Using scalpel, a 5 mm-long linear full-thickness wound was made on the skin of the prepared region (Fig 1). The wound was left to heal secondarily. Mice were then allocated as either group I receiving perilesional subcutaneous injection of 10 µg/kg



Figure 1. Mouse under anaesthesia with a full-thickness wound made on paravertebral region.

body-weight human recombinant GM-CSF (filgastrim, rHuG-CSF) 33.6 × 10⁶ IU 263 µg (Leucogyn® Kalbe Farma, batch number 2127, fab/mfg: 12 09, expiry date Nov 2011), group II receiving perilesional injection of 0.5 mg/kg body-weight dexamethasone, or group III receiving nothing as controls. Injections were given on day one to six starting on the day of wound infliction.

On the 7th day, mice were euthanized by Ketamine overdose. The wound (Fig 2), including 4 mm circumferential area extending beyond the wound margin were excised and fixed in a 10% para formaldehyde solution. Samples were prepared into paraffin blocks, sliced onto specimen slides and underwent the hematoxylin-eosin staining. Using a handy taller on 400x magnification under the microscope, number of keratinocytes and neovascularization within the samples were counted by a blinded pathologist. Data were then analyzed using the SPSS for Windows and statistical significance tested by one-way ANOVA, with a p value <0.05 considered as significant.

RESULTS

One mouse from the GM-CSF group and one from the steroid group died, making a total of 13 mice analyzed in the study: four mice in each intervention group, and five in the control group. The mean rate of keratinocyte proliferation in the GM-CSF group is 186 (± 50)



Figure 2. Clinical appearance of healed wound on day 7 after infliction of wound and daily injection of granulocyte-macrophage colony stimulating factor (GM-CSF).

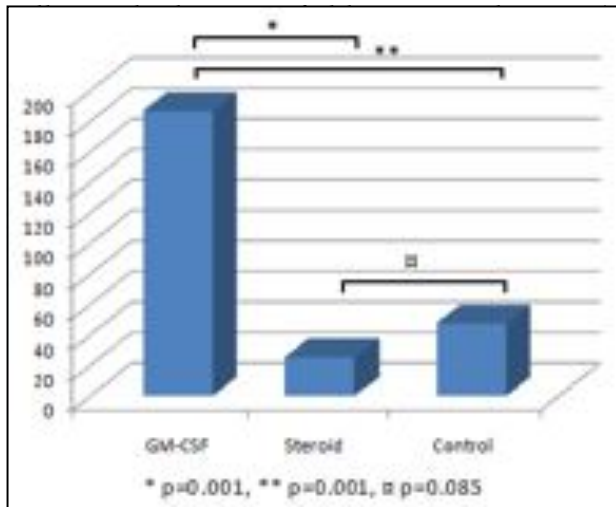


Figure 3. Mean rate of keratinocyte proliferation expressed as number of cells found per high-power field.

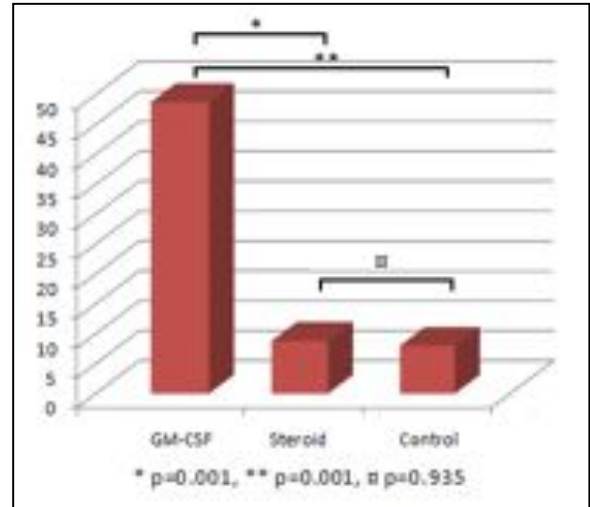


Figure 4. Mean rate of neovascularization expressed as number of new vessels found per high-power field.

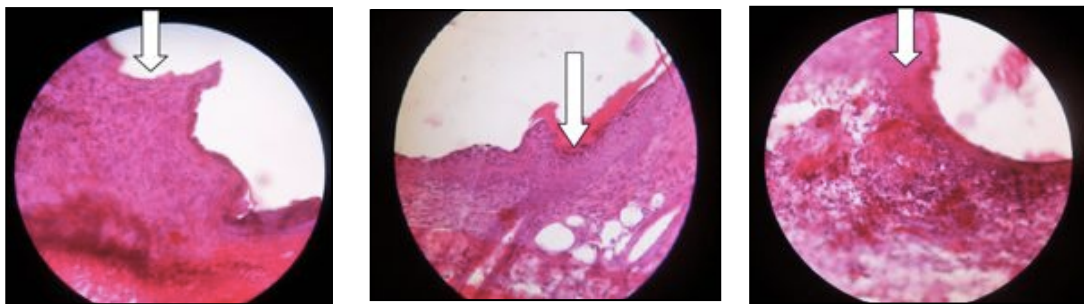


Figure 5. Histological appearance of healing skin on day-7 with hematoxyllin-eosin staining, 400 x magnification. Note the abundant number of keratinocytes and well defined dermal structures in the GM-CSF group (Left). Minimum keratinocyte numbers are found in the steroid group (middle), many inflammatory cells are seen. In the control group (Right) some keratinocytes are present but epithelial layers are not distinct.

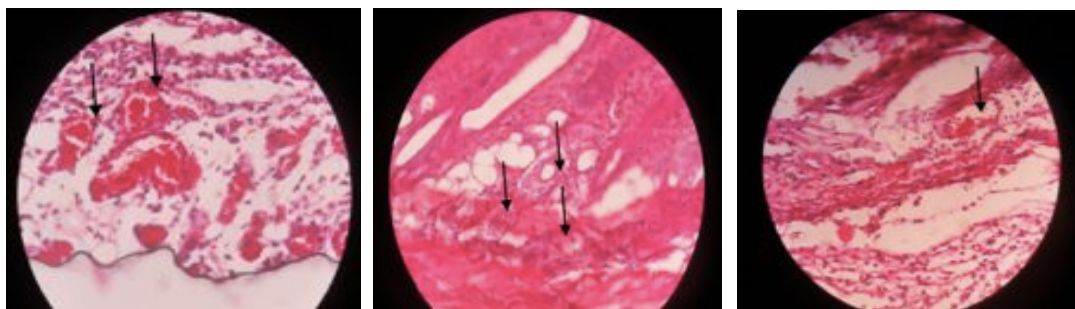


Figure 6. Histological appearance of healing skin on day-7 with hematoxyllin-eosin staining, 400 x magnification. Higher number of neovascular structures are found in the GM-CSF group (left) compared to the the steroid (middle) and control group (right).

versus steroid and control, $p=0.935$ for steroid versus control).

Histological appearance of the healing mice skin on day-7 shows higher rate of proliferation and better organization of

keratinocytes in the GM-CSF group (Fig 5 top) compared to the steroid (Fig 5 middle) and control (Fig 5 bottom) group. The control group also displays a higher number of keratinocytes compared to the dexamethasone group.

Formation of neovasculars are also far predominantly found in the GM-CSF group (Fig 6 top) compared to the minimum number of new vessel structures in the dexamethasone and control groups (Fig 6 middle and bottom).

DISCUSSION

The granulocyte-macrophage colony stimulating factor (GM-CSF) is a hematopoietic growth factor which has been widely studied for its therapeutic applications. In bone marrow transplants, GM-CSF regulates the proliferation and cell differentiation of myeloid progenitors and enhances the function of mature macrophage in inducing the release of certain cytokines including interleukin-6 and tumor necrotizing factor α into the blood stream. In cancer treatment, GM-CSF boost the number of monocytes and macrophages which lysed tumor, it acts as an effective adjuvant with low toxicity profile. The combination of GM-CSF and erythropoietin have also been shown to accelerate liver generation in experimental animals posthepatectomy^{17,18}.

GM-CSF exerts a mitogenic effect on the keratinocytes of the epidermis, as well as stimulates the migration and proliferation of endothelial cells. An accelerated rate of wound healing has been demonstrated as a result of increased proliferation of keratinocytes in laboratory animals with an overexpression of this cytokine. Not only keratinocytes are proliferating at a higher rate, under the influence of GM-CSF more vascularization and granulating tissues are also formed¹⁹. Robson and friends conducted a study observing the rate of pressure sore healing when treated with GM-CSF in combination with fibroblast growth factor beta (bFGF), GM-CSF and bFGF alone, compared to placebo²⁰. Eighty-five percent of the patient who received combined cytokine therapy gained a higher volume of tissue healing compared to the placebo group on day 35th of treatment.

Recombinant human GM-CSF (rhGM-CSF) is a biological product that has been used most successfully for clinical purpose. Sargamogastrim (rhGM-CSF obtained from mold) and molgramostim (rhGM-CSF derived from bacteria) are used to treat neutropenia

postinduction in chemotherapy for acute myelogenous leukemia. It speeds up neutrophil regeneration and decrease the occurrence of life-threatening events due to infection. The use of rhGM-CSF also exerts a favorable effect for patients undergoing autologous and allogenic bone marrow transplantation in mobilizing and engraftment progenitor cells in the blood posttransplantation.

The clinical implementation of rhGM-CSF has grown to be included as an adjuvant in cancer therapy and chemoradiation complication such as mucositis, stomatitis, and diarrhea. Topical application of rhGM-CSF accelerates the process of wound healing in forming granulation tissue. Intradermal injection of rhGM-CSF increases the rate of keratinocyte proliferation, thickens the epidermal layer and hastens the rate of overall healing. Short-term use of rhGM-CSF in subcutaneous tissue effectively promote the growth of new blood vessels. It as also demonstrated a positive effect in improving Chron's disease symptoms¹⁸.

In this experiment, rhGM-CSF and dexamethasone were administered subcutaneously in the intentionally made wound on lab mice. rhGM-CSF and dexamethasone were given from day one to day six postwounding, with the respective dose of 10 μ g/kg body weight (BW) and 10 mg/kg BW diluted with sterile distilled water to adjust dose according to animals body weight. At 10 μ g/kg BW, subcutaneous administration of rhGM-CSF mobilizes granulocyte progenitor cells (cells bearing CD 34+) from the bone marrow to peripheral circulation starting on day 4 up to the 7th day. On the other hand, a 10 mg/kg BW dexamethasone injection inhibits the release of natural GM-CSF^{15,21}.

Histologically, keratinocytes and neovascular structures are viewed under light microscope (400x magnification) with hematoxylin-eosin staining and cells counted using the handy taller. Keratinocytes are distinguishable from its keratinized structure factor (PDGF) which together with the transforming growth factor beta (bTGF), fibroblast growth factor (FGF), and insulin like

with dark blue nuclei. New growth of vascularization are identified from the erythrocyte cells trapped within the lumen. Neovasculars are differentiated from mature blood vessels from the less developed endothelial structures. In this study, the number of keratinocytes on day 7 post intervention and therapy: in the rhGM-CSF experimental animals are more than threefold higher than the control group (186 ± 50 cells/hpf v.s. 47 ± 16 cells/hpf), and those given steroid displayed half the number of keratinocytes than that of controls (25 ± 10 cells/hpf v.s. 47 ± 16 cells/hpf). Under the microscope, keratinocytes are better aligned in the basal layer -and displays more intracell volume- reaching up to the transitional layers in the rhGM-CSF group compared to steroid and controls. These results are in line with what is expected from the evidence-based theories that 1) GM-CSF hastens wound healing, and 2) steroids hinders healing; although point number two did not reach statistical significance ($p=0.08$), it shows a noteworthy pattern. As stated in the literature review, fluticasone propionate and budesonide are more potent inhibitors of GM-CSF than dexamethasone¹⁵ hence if we were to use those agents instead of dexamethasone the difference in the keratinocytes proliferation might be expressed more solidly. Each mouse releases endogenous GM-CSF immediately after wounded, and because the level is not measured quantitatively we cannot conclude for certain that the decrease in keratinocytes and slower healing in the dexamethasone group is due to lower GM-CSF level.

Another factor that was not measured is the keratinocyte mitosis rate in the in follicular basal epidermal layer. An experiment was done by administering GM-CSF antagonist to double transgenic Tg2-Ant mice with a GM-CSF over-expression, and to wildtype mice as controls². GM-CSF antagonist was shown to suppress GM-CSF-dependent keratinocyte hyperproliferation in the wildtype mice, which did not occur in the transgenic mice relative to the control group. Further immunohisto-chemistry staining on the transgenic mice keratinocytes mitosis markers confirmed the result.

The migration and proliferation of keratinocytes from wound edges, together with the increased synthesis of metalloproteinase (MMP) will further mobilize keratinocytes to cover the wound surface. When keratinocytes from all edges meet in the center, epithelial proliferation decreases three- to four-times slower than normal until the function and morphology of epidermal cells will return. Continuous expression of growth factor, combined with abnormal wound bed environment such as reduced moisture level, ischemia, and repetitive trauma will constantly stimulate proinflammatory cells activity. Excessive neutrophilic infiltration followed by the influx of macrophages will initiate the release of $TNF\alpha$ and $IL-1\beta$. These cytokines are fibroblast and inflammatory cells chemoattractant which sustains MMP. When MMP is inhibited, growth factor and growth factor receptors are reduced and this slows down the process of wound healing¹⁹.

Similar results are also found when neovascular structures of the mice in intervention and control groups are measured. New formation of blood vessels in the rhGM-CSF group are around six-fold higher (48 ± 17 vessels/hpf) than those found in the steroid and control groups (8 ± 6 vessels/hpf and 7 ± 5 vessels/hpf respectively). This further establish the positive effect of rhGM-CSF in enhancing wound healing, in line with the literature which stated that GM-CSF plays an important factor for endothelial proliferation^{8, 22}. Deficiency in GM-CSF alters the vascular collagen matrix composition which support an important factor for GM-CSF to preserve blood vessels integrity and immunity. However, after wound occurs various cytokines are released and the overexpression of one type of cytokine will stimulate the release of other cytokines. The synergistic effect of these cytokines will play their role in wound healing.

Trauma induces genetic expression of vascular endothelial growth factor (VEGF), macrophage, and keratinocytes. VEGF is a potent stimulator for angiogenesis, and a decrease in its amount will hinder healing process. The same goes for other growth factor hormones such as the platelet derived growth

growth factor (IGF) control the formation of macrophage-dependent fibroblasts¹⁹. The minimal neovascularization found in the steroid group may be due to the decrease in macrophage colony because of dexamethasone antagonistic effect on various cytokines (VEGF, bTGF, PDGF, IGF and FGF) all of which are important growth factors in the process of wound healing. The exact mechanism of this antagonistic influence of steroids warrant a further research.

As an addition, the increase in number of progenitor cells in circulating peripheral blood post rhGM-CSF induction -which can be isolated and purified- is a study prospect on the matter of "the applicability of peripheral blood progenitor cells for cell-targeted therapy". The cell's unique characteristic of differentiation and self-renewal are useful to treat cases of chronic skin ulcers, unhealing wounds, or inherited dermatologic problems such as epidermolysis bulosa.

CONCLUSION

Granulocyte macrophage colony stimulating factor (GM-CSF) hastens wound healing in wildtype mice by increasing the rate of keratinocyte proliferation and neovascular formation, while dexamethasone has a tendency to hinder wound healing because it acts as a GM-CSF inhibitor. Further study to elucidate the mechanism of GM-CSF effect in wound healing may be done using transgenic mice with immunohistochemistry study to evaluate mitotic markers of keratinocytes and neovasculars.

Eddy Sutrisno, MD.

Plastic Surgery Division

Adam Malik General Hospital, Medan. Universitas Sumatera Utara

Accuplastclinic@yahoo.com

REFERENCES

- Gurtner CG. Wound Healing : Normal and Abnormal. In: HC T, ed. *Grabb Plastic Surgery*. 6th Ed. Philadelphia: Wolters Kluwer-Lippincott William and Wilkins; 2007:15-22.
- Mann A, Niekisch K, Schirmacher P, Blessing M. Granulocyte-macrophage colony-stimulating factor is essential for normal wound healing. *J Invest Dermatol Symp Proc*. Sep 2006;11(1):87-92.
- Regauer S, Compton CC. Cultured keratinocyte sheets enhance spontaneous re-epithelialization in a dermal explant model of partial-thickness wound healing. *J Invest Dermatol*. Sep 1990;95(3):341-346.
- Stricklin GP, Li L, Nanney LB. Localization of mRNAs representing interstitial collagenase, 72-kda gelatinase, and TIMP in healing porcine burn wounds. *J Invest Dermatol*. Sep 1994;103(3):352-358.
- Martin P. Wound healing--aiming for perfect skin regeneration. *Science*. Apr 4 1997;276(5309):75-81.
- Stadelmann WK, Digenis AG, Tobin GR. Impediments to wound healing. *Am J Surg*. Aug 1998;176(2A Suppl):39S-47S.
- Baker EA, Leaper DJ. Proteinases, their inhibitors, and cytokine profiles in acute wound fluid. *Wound Repair Regen*. Sep-Oct 2000;8(5):392-398.
- Mann A, Breuhahn K, Schirmacher P, Blessing M. Keratinocyte-derived granulocyte-macrophage colony stimulating factor accelerates wound healing: Stimulation of keratinocyte proliferation, granulation tissue formation, and vascularization. *J Invest Dermatol*. Dec 2001;117(6):1382-1390.
- Hunt KT. Wound Healing. In: Doherty MG, ed. *Current Surgical Diagnosis and Treatment*. 12th Ed. USA: McGraw-Hills; 2003:75-87.
- Ting AE, Mays RW, Frey MR, Hof WV, Medicetty S, Deans R. Therapeutic pathways of adult stem cell repair. *Crit Rev Oncol Hematol*. Jan 2008;65(1):81-93.
- Brem H, Balledux J, Bloom T, Kerstein MD, Hollier L. Healing of diabetic foot ulcers and pressure ulcers with human skin equivalent: a new paradigm in wound healing. *Arch Surg*. Jun 2000;135(6):627-634.
- Stagno F, Guglielmo P, Consoli U, Fiumara P, Russo M, Giustolisi R. Successful healing of hydroxyurea-related leg ulcers with topical granulocyte-macrophage colony-stimulating factor. *Blood*. Aug 15 1999;94(4):1479-1480.
- Kaplan G. Recent advances in cytokine therapy in leprosy. *J Infect Dis*. Mar 1993;167 Suppl 1:S18-22.
- Ure I, Partsch B, Wolff K, Petzelbauer P. Granulocyte/macrophage colony-stimulating factor increases wound-fluid interleukin 8 in normal subjects but does not accelerate wound healing. *Br J Dermatol*. Feb 1998;138(2):277-282.
- Adcock IM, Nasuhara Y, Stevens DA, Barnes PJ. Ligand-induced differentiation of glucocorticoid receptor (GR) trans-repression and transactivation: preferential targeting of NF-kappaB and lack of I-kappaB involvement. *Br J Pharmacol*. Jun 1999;127(4):1003-1011.
- Al Homsy AS. Cytokine Modulation of Hematopoietic Stem Cell Phenotype. In: Ho Anthony D, Haas R, Champlin ER, eds. *Hematopoietic Stem Cell Transplantation*. New York-Basel: Marcel Dekker; 2000:47-61.
- Vassiliou I, Lolis E, Nastos C, et al. The combined effect of erythropoietin and granulocyte macrophage colony stimulating factor on liver regeneration after

- major hepatectomy in rats. *World J Surg Oncol.* 2010;8:57.
18. Ghosh KP, Bhardwaj D, Karnik R. Human Granulocyte-Macrophage Colony Stimulating Factor: The Proteins and Its Current and Emerging Applications. *Indian J of Biotech.* 2007;6:435-448.
 19. Cornelissen LH. Wich Molecules of Initial Phase of Wound Healing may be Used as Markers for Early Detection of Skin Damage? Eindhoven.: Technische Universiteit; 2004.
 20. Robson MC, Hill DP, Smith PD, et al. Sequential cytokine therapy for pressure ulcers: clinical and mechanistic response. *Ann Surg.* Apr 2000;231(4): 600-611.
 21. Lane AT, Law P. Mobilization of Pheripheral Blood Stem Cells from Normal Donors. In: Ho AD, Haas R, Champlin ER, eds. *Hematopoietic Stem Cell Transplantation.* New York, Basel: Marcel Dekker; 2000:333-351.
 22. Ebner K, Bandion A, Binder BR, de Martin R, Schmid JA. GMCSF activates NF-kappaB via direct interaction of the GMCSF receptor with IkappaB kinase beta. *Blood.* Jul 1 2003;102(1):192-199.
 - 23.