Does priming granulocyte-macrophage colony-stimulating factor increase tissue CD4/CD8 ratio in impaired flap healing?

Bayram KIRAN[1], Selma SÖNMEZ ERGUN[5], Faruk SÜZERGÖZ[3], Özlem SU[6], Ali Osman GÜROL[3,4], Bilge BİLGİÇ[2]

[1]Departments of Microbiology, Division of Virology and Basic Immunology,
[2]Department of Pathology, Istanbul Medical Faculty,
[3]Department of Immunology,
[4]Metabolism and Diabetes Research and Application Unit, Institute of Experimental Medicine (DETAE), Istanbul University, and
[5]Department of Plastic and Reconstructive Surgery, [6]Department of Dermatology, Vakif Gureba Hospital, Istanbul, Turkey

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Summary

ABSTRACT

Background: The presence of an intact T-cell immune system is essential for a normal wound healing. Glucocorticoids impair cellular immune response by inhibiting T-cell proliferation, but granulocytemacrophage colony-stimulating factor (GM-CSF) which is a proinflammatory cytokine leads to immune stimulation by increasing the levels of circulating CD4 and CD8 cells. The purpose of this study was to elucidate the role of lymphocytes in impaired wound healing model by using immunohistochemistry.

Methods: 72 Wistar albino rats (200 to 240 g) were divided into three groups: Group I (given saline + flap elevation); Group II (given systemic methylprednisolone + saline + flap elevation); Group III (given systemic methylprednisolone + GM-CSF + flap elevation). Samples fixed in 10 % formaline and embedded in parafin were used. The sample sections were evaluated immunohistochemically using monoclonal antibodies for CD4 and CD8.

Results: CD4 and CD8 staining of histologic sections in GM-CSF-treated group III were better than those of group II, although they all remained worst than those of group I ($p > 0.05$).

Conclusions: The results obtained in this study, suggest the possiblity to abrogate the immunesuppressive effects of methylprednisolone by GM-CSF injection. To restore lymphocyte function using proinflammatory cytokines is important for wound healing and prevention of immunosuppression.

Key words: Flap healing, immunohistochemistry, GM-CSF, CD4, CD8

Granülosit-makrofaj koloni-stimülan factor uygulanması bozulmuş flep iyileşmesinde doku CD4/CD8 oranını arttırıyormu?

Özet

Amaç: Yara iyileşmesinde T hücre bağımlı immün yanıtın sağlıklı olması esansiyel öneme sahiptir. Glukokortikoidler T hücre proliferasyonunu inhibe ederek hücresel yanıtı bozmalarına karşın bir proinflamatuar sitokin olan granülosit-makrofaj koloni stimülan faktör (GM-CSF) dolaşımdaki CD4/CD8 oranını arttırarak immün stimülasyon gerçekleşir. Bu çalışmada bozulmuş yara iyileşmesi modelinde lenfositlerin rolünün immunhistokimyasal olarak ortaya konulması amaçlanmıştır.

Gereç - Yöntem: 72 adet Wistar albino rat (200 – 240 g arası) üç gruba ayrılmıştır: I Grup (Serum fizyolojik + flep kaldırma); II Grup (Sistemik metilprednizolon uygulaması + serum fizyolojik + flep kaldırma); III Grup (Sistemik metilprednizolon uygulaması + GM-CSF + serum fizyolojik + flep kaldırma). Doku örenkleri % 10' luk formalinde fikse fiksasyonun ardından parafine gömüldükten sonra kullanılmıştır. Örneklerden alınan kesitler CD4 ve CD8 monoklonal antikorları ile boyandıktan sonra immünohistokimyasal olarak incelenmiştir.

Bulgular: I Gruptaki kesitlerden daha kötü olmasına karşılık CD4 ve CD8 ile boyanma III Gruba ait histolojik kesitlerde II Gruba ait olanlardan daha iyi boyanmıştır ($P<0.05$).

Sonuç: Elde edilen veriler GM-CSF enjeksiyonu ile metil prednizolonun immünsüpresif etkisinin ortadan kaldırılmasının mümkün olabileceğini göstermektedir. Lenfosit fonksiyonlarının onarımı için proinflamatuar sitokinler kullanılmasının yara iyileşmesinde ve immünsüpresyondan korunmada önem taşıdığına inanmaktayız.

Anahtar Kelimeler: Flep iyileşmesi, immünohistokimya, GM-CSF, CD4, CD8

INTRODUCTION

Wound healing requires complex interactions between a variety of cell types in order to restore tissue integrity. Prolonged and complicated wound healing may occur in patients with suppressed or deficient immune function. An understanding of the interactions between the immune system and healing responses would enable the design of pharmaceuticals that modulate these interactions. Modulating levels of cytokines and growth factors are known to participate in initiating and sustaining the time-dependent phases of wound repair. Priming tissue to be incised is a new approach to wound healing. Pretreatment of tissue with an appropriate proinflammatory cytokine creates an environment in which the components of the tissue repair process are activated prior to incision (1-7).

The main modulators of tissue repair are growth factors which besides several physiological processes play a significant role in wound healing by inducing mitogenic activity as well as migration of leucocytes and fibroblasts into injured tissue. Several clinical trials attempting to accelerate wound healing have involved application of exogenous recombinant growth factors to chronic wounds. One of those growth factors is granulocyte-macrophage colony-stimulating factor (GM-CSF) which is a proinflammatory cytokine, and chemotactic for macropages and neutrophils that induces the formation of early wound elements necessary for fibroblast migration. It has been shown in experimental and clinical studies that incisional wounds where wound healing has been impaired by methylprednisolone injection, infected acute and chronic wounds heal significantly faster than the controls when GM-CSF is locally applied (5, 8-10).

In this study, the role of locally administered GM-CSF on wound healing was investigated immunohistochemically using monoclonal antibodies for CD4 and CD8 in rats with impaired wound healing due to systemic glucocorticoid administration.

METHODS

Animals

Wistar albino rats weighing 200-240 g were purchased from the Institute of Experimental Medicine (DETAE) of Istanbul University and kept on a standart laboratory diet and tap water. The experimental procedures and care of animals were in accordance with the Istanbul University's Local Ethical Committee for Experimental Animals.

Experimental Protocol

Seventy two rats were randomly divided into three groups:

Group I [(Control group), (n=24)], given saline (3 cc perilesional subcutaneous saline injections) + flap elevation;

Group II (n: 24), given systemic methylprednisolone [(30 mg/kg, an intramuscular injection 2 days before flap elevation) (Prednol-L, Mustafa Nevzat® Pharmaceuticals, Istanbul, Turkey)] + given saline (same as group I) + flap elevation;

Group III (n: 24), given systemic methylprednisolone (same as group II) + given GM-CSF [(200 μg/kg/day in 3 cc phosphate-buffered saline solution, perilesional subcutaneous GM-CSF injections) (Leucomax, Novartis® Pharmaceuticals, Istanbul, Turkey)] + flap elevation. Experimental protocol is shown in Table 1.

Table 1: Experimental protocol

Groups	Priming with saline*	Priming with GM-CSF**	Methylprednisolone injection***	Number of rats
Group I	7x3ml/day	(-)	(-)	24
Group II	7x3ml/day	(-)	1x30 mg/kg/day	24
Group III	(-)	7x200μg/kg/day	1x30 mg/kg/day	24

*, ** Given subcutaneously for 2 days before surgical procedure (surgical procedure included elevation of modified McFarlane flaps (6x3 cm) and suturing of the flaps to the recipient bed following elevation) and 5 days after surgical procedure.

*** Given intramuscularly 2 days before surgical procedure.

Injectional and Surgical Procedure

After the induction of general anesthesia by 40 mg/kg intraperitoneally thiopentone sodium (Pental Sodyum, Ibrahim Ethem Ulagay® Pharmaceuticals, Istanbul, Turkey), dorsal regions were shaved, the animals were placed in prone

position and then a rectangle (6x3 cm in size) was drawn onto the backs of the rats and first subcutaneous injections were applied to the flap incision lines. In group II and group III, impaired healing was induced by an intramuscular injection of methylprednisolone (30 mg/kg). After recovery from anesthesia, animals were housed individually and given food and water ad libitum.

Injections described above in each group were applied 2 days before the operation and on 1st, 2nd, 3rd, 4th, and 5th postoperative days.

After subcutaneous injections applied to the flap incision lines for two days, the rats were anaesthetized again, and modified McFarlane flaps (6x3 cm) (Fig. 1) were elevated just deep to the panniculus carnosus in a relatively avascular plane using blunt dissection. Following elevation, flaps were returned to their recipient beds and continously sutured into place using 4/0 silk sutures. This suture was removed before sampling.

Parafin-embedded tissue samples obtained from twelve rats in each group were sacrificed on 7th and 14th days postoperatively.

Tissue Sampling

Samples which were fixed in 10 % formaline and embedded in parafin were used. Four-to five-micrometer-thick sections were subjected to immunohistochemistry. The samples were evaluated immunohistochemically using monoclonal antibodies for CD4 and CD8. CD4 (anti-rat CD4, Cymbus Biotechnology CBL 1506, Lot: PC1B368N) and CD8 (anti-rat CD8, Cymbus Biotechnology CBL 1507, Lot: 1507110L) were prepared at a dilution of 1:10 and 1:50, respectively. Antigen retrieval using pressure cook was performed. The sections were incubated with the primary antibodies at room temperature for 2 hours. All slides were subjected to light microscopic examination and evaluated by two pathologists in a blind fashion. The numbers of lymphocytes were assessed on the arbitrary scale of 0-3 (0: no lymphocytes; 3: very dense infiltrate of lymphocytes).

Statistical Analysis

All data are presented as mean standart±deviation in the text and figures. Statistical significance was determined by Kruskal-Wallis test where necessary. A p-value less than 0.05 were considered significant.

Results

There were significant differences in CD4 and CD8 staining of histologic sections among the groups (Fig. 1 and 2). There were significant differences in CD4 and CD8 staining of histologic sections between the group II and group I and III ($P<0.016$) on day 7 and on day 14 (Table 2, 3). Although CD4 and CD8 staining of histologic sections in GM-CSF-treated group III were better than those of group II, they all remained worst than those of group I ($P<0.05$).

Figure 1: CD4 staining for flap elevation + saline; flap elevation + methylprednisolone + saline; flap elevation + methylprednisolone + GM-CSF groups of histologic sections on day 7 (A, B, C x200) and 14 (D, E, F x200) post-operatively.

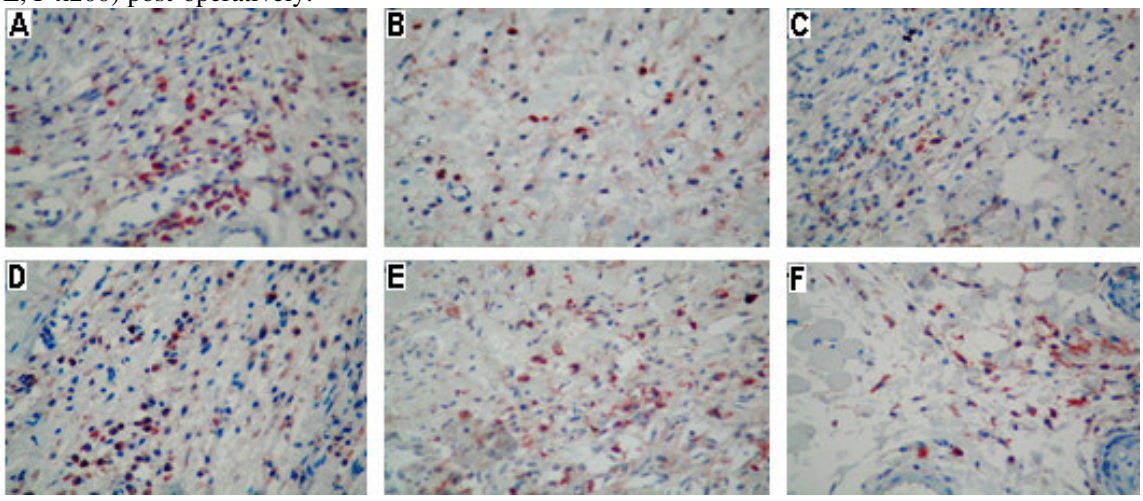

Figure 2: CD8 staining for flap elevation + saline; flap elevation + methylprednisolone + saline; flap elevation + methylprednisolone + GM-CSF groups of histologic sections on day 7 (A, B, C x200) and 14 (D, E, F x200) post-operatively.

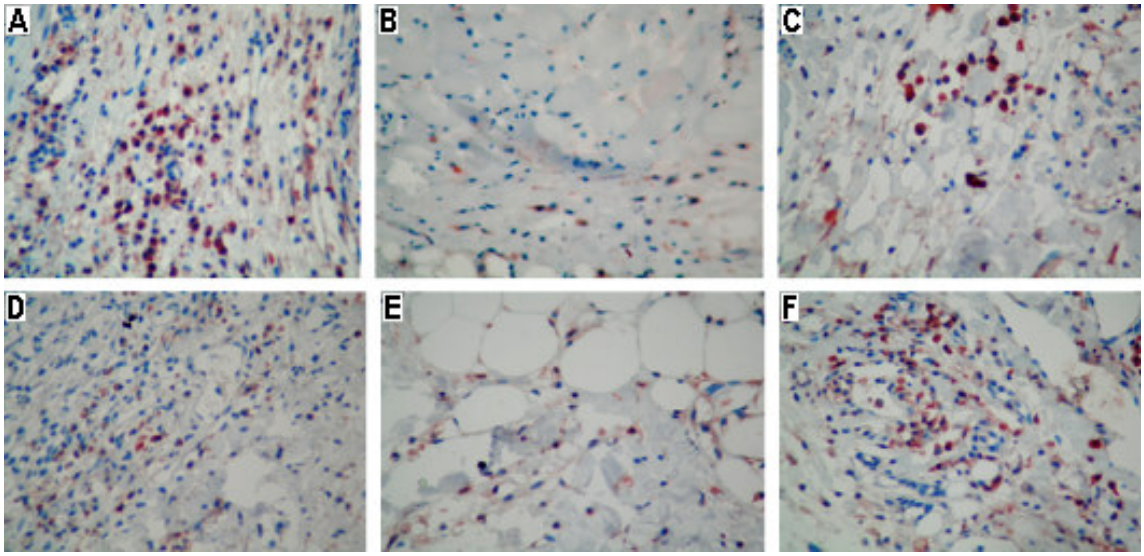

Table 2: CD4 and CD8 staining of histologic sections on day 7 postoperatively.

Variables	Groups (n:12)	Median	Minimum-Maximum	K-W*	Two-tailed significance
CD4	I	2	2-3	23.197	p<0.0001
	II	1	0-2		
	III	2	1-3		
CD8	I	2	2-3	21.132	p<0.0001
	II	1	0-2		
	III	2	1-3		

*Kruskall-Wallis

Table 3: CD4 and CD8 staining of histologic sections on day 14 postoperatively.

Variables	Groups (n:12)	Median	Minimum-Maximum	K-W*	Two-tailed significance
CD4	I	2	2-3	17.53	P<0.0001
	II	1	0-1		
	III	2	1-2		
CD8	I	2.5	1-3	15.06	P<0.001
	II	1	0-2		
	III	2	1-3		

*Kruskall-Wallis

Discussion

It has been reported that GM-CSF is chemotactic and mitogenic for fibroblasts and endothelial cells in vitro. A stimulatory effect on fibroplasia and collagen deposition by GM-CSF administered locally was also suggested by the increased mechanical strength of incisional wounds in immunosuppressed rats. By manipulating the inflammatory and early proliferative phases of wound healing with GM-CSF, it may be possible to accelerate acute wound repair and shift the wound healing trajectory to the left (1, 8, 10). The cellular arm of the system, composed of neutrophils, macrophages and lymphocytes, plays a major role in wound repair. Both in vitro and in vivo studies have demonstrated that the presence of both macrophages and T lymphocytes at the wound site is essential for a normal healing process (11).

The presence of macrophages is essential for the initiation and maintenance of wound fibroblast activity. T-cells do not appear to be required for

the initiation of the healing process, and healing can progress in the absence of T-lymphocytes, but the presence of an intact T-cell immune system is essential for a normal outcome (12-14). T-lymphocytes regulate fibroblast migration, replication, and collagen synthesis via secreted lymphokines. In the process of wound healing there is a well defined balance of both stimulatory and inhibitory lymphokines regulating the orderly cellular activity (15). An imbalance of these factors caused by a global depletion of CD4, CD8, or CD4 and CD8 lymphocytes removes the regulatory influence controlling the activity of fibroblasts and macrophages. This ultimately influences the collagen and proteoglycan content of the extracellular matrix of the wound, resulting in wounds with altered biomechanical properties. In vivo use of lympholytic agents such as steroids, cyclosporin A, or anti Thy 1.2 monoclonal antibody markedly impair wound breaking strength and hydroxyproline deposition. Conversely, lymphotrophic agents such as vitamin A, arginine, IL-2, HGF, and GM-CSF leads to an increase in both wound breaking strength and wound collagen deposition (16-21). Direct evidence for lymphocytes involvement in the process of wound healing is provided by studies examining the effects of in vivo depletion on wound healing and the cellular response within the wounds is measured by immunostaining as was done in our study. CD4 and CD8 cells provide the maintenance of cellular immune response in an optimal level by controlling each other's function via negative feedback T-lymphocyte participation in wound healing. Thus it is a finely tuned mechanism dependent on the existing balance between T-lymphocyte subpopulation. Therfore CD4/CD8 ratio (which is 1.7-2.5 normally) is very important for cellular immunity (22). Lowering in this ratio leads to cellular immune deficiency. Glucocorticoids impair cellular immune response by lowering the levels of circulating CD4 and CD8.

There are some studies about the treatment with methylprednisolone resulted in significant depletion of T-lymphocytes in the periferal blood as demonstrated by flow cytometry. Ergun et al. previously reported that in vivo T-lymphocyte depletion significantly impairs wound healing, as assessed by breaking strength and hydroxyproline content (16). In another study, it is emphasized that human wound-associated lymphocyte populations are modulated during healing; the increase in numbers of CD8+ T-suppressor lymphocytes is in accordance with previous animal data, indicating a role for these cells in downregulating healing as the wound closes (12).

The most likely explanation for the observed impairment in wound healing is the marked depletion of T-lymphocytes. GM-CSF has been shown to stimulate granulocyte and macrophage cell lineage proliferation, properties which may be important to the wound healing process. In addition, other properties such as an ability to increase the oxidative capacities of cells and the observation that GM-CSF is chemotactic for neutrophils may also positive implications for wound healing. GM-CSF leads to immunomodulation by increasing levels of circulating CD4 and CD8 cells (14, 23-32). As demonstrated immunohistochemically in this study, it may be possible to abrogate the immunesuppressive effects of methylprednisolone by GM-CSF injection.

Chronic, nonhealing wounds represent a major clinical challenge to practically all disciplines in modern medicine. Perturbation in the host immune system can manifest as complications in wound healing, as seen in patients with immunosuppression, steroid use, diabetes, or malnutrition. These patients often have abnormalities of T-lymphocyte function and this may result in an effective downregulation.

In the future it may be possible to manipulate the T-lymphocyte system (locally or systemically, depending on the clinical situation) at specific stages of wound healing to enhance repair in patients who are at risk of impaired healing. This suggests that in certain disease states deficient wounds can be treated by exogenous growth factors such as GM-CSF to accelerate healing wound (9, 17, 19, 33-36).

In conclusion, to restore lymphocyte function using proinflammatory cytokines before injury is important for wound healing and prevention of immunosuppression. This new approach may be imporant therapeutic implications for preparation of skin sites before surgical procedures on risky patients.

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Correspondence: Bayram KIRAN, PhD
Department of Microbiology, Division of Virology and Basic
Immunology, Istanbul Univeristy, Istanbul Medical Faculty,
Capa- Istanbul, Turkey.
E-mail: drbayramkiran@yahoo.com
Phone : + 90 212 414 2000 (ext. 32144), Fax : + 90 212 635 2582