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The Decrease in the Histamine Content of Irradiated Wound Tissue by GM-CSF: A Comparison Between Histamine and Ascorbic Acid

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The Decrease in the Histamine Content of Irradiated Wound Tissue by GM-CSF: A Comparison Between Histamine and Ascorbic Acid

Summary : *As a result of preoperative radiotherapy, impaired or delayed wound healing may be a frequent clinical problem. Wound healing is characterized by synthesis of collagen. Histamine and ascorbic acid have important effects on collagen synthesis. In this controlled, randomized study; it was aimed to evaluate the effect of GM-CSF applied topically on the preoperatively irradiated wound healing in the context of ascorbic acid and histamine content. Half the rats were irradiated as 8 Gy total body irradiation and 2 days after radiotherapy, they were randomized into four groups: Group 1-control, group 2-GM-CSF applied, group 3-irradiated control group, group 4-irradiated and GM-CSF applied group. 11 days after radiotherapy tissue samples were taken. Histamine content of the wound was found to be increased 320% by irradiation (p=0.002) and decreased 81% by drug administration in the irradiated wound (p=0.002). The ascorbic acid content was decreased 51% by irradiation and GM-CSF made no change on its level. The results show that GM-CSF diminishes the increased histamine content of the irradiated wound. Further investigations are required to determine the mechanisms involved.*

Key Words: *Wound healing, radiotherapy, histamine, ascorbic acid, GM-CSF*

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GM-CSF ile Işınlanmış Yara Dokusu Histamin Miktarındaki Azalma: Histamin ve Askorbik Asit Arasındaki Kıyaslama

Özet : *Preoperatif radyoterapinin bir sonucu olan bozulmuş veya gecikmiş yara iyileşmesi sıklıkla klinik bir problem olmaktadır. Yara iyileşmesi kollajen sentezi ile karakterizedir. Histamin ve askorbik asit kollajen sentezinde önemli etkilere sahiptirler. Bu kontrollü, randomize çalışmada preoperatif ışınlanmış yara dokusunda topikal olarak uygulanan GM-CSF'in yara iyileşmesi üzerine etkisinin askorbik asit ve histamin miktarı açısından değerlendirilmesi amaçlanmıştır. Deneklerin yarısına 8 Gy'lik tüm vücut ışınlaması uygulanıp ışınlamadan 2 gün sonra 4 gruba randomize edildi: Grup 1-kontrol, grup 2-GM-CSF uygulanan grup, grup 3-ışınlanan kontrol, grup 4-ışınlanan ve GM-CSF uygulanan grup. Işınlamadan 11 gün sonra doku örnekleri alındı. Işınlama ile yara dokusu histamin miktarının %320 arttığı (p=0.002) ve ışınlanmış dokuya ilaç uygulamasıyla %81 azaldığı (p=0.002) bulundu. Işınlama ile askorbik asit düzeyinin %51 azaldığı, GM-CSF uygulamasının bu düzeyi değiştirmediği tespit edildi. Bu sonuçlar GM-CSF' in ışınlama ile artan yara dokusu histamin miktarını azalttığını göstermektedir. İlgili mekanizmaların belirlenmesi için ileri çalışmalara gerek vardır.*

Anahtar kelimeler : *Yara iyileşmesi, radyoterapi, histamin, askorbik asit, GM-CSF*

INTRODUCTION

Radiation therapy is frequently combined with surgery pre- or postoperatively in the management of malignancies. As a result, impaired or delayed healing of wound in irradiated tissue may frequently be present in clinical problems[1]. A number of autocoids and growth factors play a role in inflammation and possibly in the healing process. Several investigators have established the key role of growth factors in regulating the recruitment of leukocyte, monocyte and fibroblasts into wound area[2,3]. Blood-borne elements are crucial to proper wound healing[3].

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Granulocyte macrophage-colony stimulating factor (GM-CSF) is one of the specific glycoproteins necessary for the proliferation of haematopoietic progenitor cells. In addition to its proliferative action, GM-CSF also stimulates various functional activities of mature granulocytes, macrophages and eosinophils[4]. GM-CSF is synthesized by macrophages, T lymphocytes, fibroblasts, endothelial cells and keratinocytes. The results in the current literature suggest that GM-CSF may be the physiological component of a growth cascade within healing wounds[5]. GM-CSF enhances keratinocyte proliferation and differentiation and induction of neovascularization[6].

Mast cells participitate in all stages of wound healing. Healing of wounds is characterized by synthesis of connective tissue, the major component of which is collagen[7]. The sensitivity of mast cells to irradiation was implied from observations that mast cell-derived autocoids, eg. histamine, prostaglandins are released in surrounding tissues following exposure to gamma irradiation[8]. There are some data which indicate that histamine stimulate at low doses and at high doses inhibit collagen synthesis[9]. Exposure of the body to x-rays and gamma rays produces oxygen free radicals which damage proteins, lipid and nucleic acid[10]. Ascorbic acid is a natural antioxidant compound and exerts a radioprotective effect[11]. Ascorbic acid is also important in wound healing due to its essential role in collagen synthesis[12].

Data that support the therapeutic efficacy of haematopoietic growth factors on wound healing are very limited and only anecdotal. We found no studies that evaluate the effect of GM-CSF on wound healing according to histamine and ascorbic acid content. In this study, it was aimed to evaluate the effect of GM-CSF on the ascorbic acid and histamine content of the irradiated skin wound for the first time in the literature.

MATERIALS AND METHODS

The protocol for the study was approved by the Ethical Committee of Gazi University Faculty of Medicine Animal Breeding and Research. Twenty-eight adult (230-350 mg) male, Wistar-Albino rats were divided into the following four groups:

Non-irradiated- Group 1 (n=7)-Control
Group 2 (n=7)-GM-CSF administered
Irradiated- Group 3 (n=7)-Control
Group 4 (n=7)-GM-CSF administered

The rats in groups 3 and 4 were anesthesized by intraperitoneal injection of Ketamin HCl, 50 mg/ kg, on the day of irradiation. Recombinant human granulocyte macrophage-colony stimulating factor (rHuGM-CSF, Molgramostim,Leucomax®, Novartis/ Schering-Plough, Switzerland), 15 µg/ wound, 0.1 mL total volume, was applied to incisions with tuberculin syringes at the time of wounding subcutaneously between two wound edges of the rats in group 2 and group 4 two days after irradiation. The animals were housed individually in a controlled environment and fed with standard rat chow and water.

Total Body Irradiation (TBI)

A cobalt 60 teletherapy instrument (Theratron 780 C) with a radiation field size of 30x30 cm was used to deliver a single peak whole body dose of 8 Gy to a depth of 3 cm as described[13]. A single anterior field was used for irradiation and four animals were treated at a time. The skin surface received a dose of only 8.8 Gy, a dose with minimal effects on dermal fibroblasts. At a tissue depth of 5 cm, less than a 10% decrease in total-body dosage was seen[14]. The Cobalt 60 unit was calibrated with a Farmer Ionization Chamber (PTV Unidose Dosimeter, Nuclear Enterprises Ltd., Beenham Reading, UK) (0.6 ml). A ±3% uncertainity in absorbed dose was estimated.

Production of Linear Surgical Incision

Irradiation was carried out 2 days before wounding because previous work revealed this interval to result in the most significant wound impairment composed to any other interval up to 3 weeks prior to wounding[13]. Rats were anesthesized with Ketamine HCl (50 mg/ kg), and their dorsal region was shaved and

cleaned. A linear surgical wound 4 cm. long was produced through the dorsal skin in fully anesthesized animals[13]. The wounds were closed with four surgical clips (4/0 silk). The entire procedure was carried out under aseptic conditions. On day 9 post-wounding, animals were killed with overdose anesthetics and incisional tissue samples were taken to measure the level of histamine and ascorbic acid content.

Measurement of Histamine Content of Tissue

Histamine content of the skin was measured using the method reported by Shore et al.[15]. Tissue samples were homogenized with perchloric acid, the homogenate was centrifuged and the supernatant was used for analysis. Histamine was determined by fluorometric method. The eluate was derivatized with o-phthaldehyde. Fluorescence intensity was at 450 nm with excitation with 360 nm in a spectrofluorometer[15].

Measurement of Ascorbic Acid Content of Tissue

Skin ascorbic acid contents were estimated by the method of Berger et al.[16]. Briefly, tissue samples were homogenized in ice-cold perchloric acid-etilendiamintetraacetic acid (PCA/EDTA) (1 g tissue plus 9 times PCA/ EDTA) in tissue homogenizer. After centrifugation at 15000 rpm for 3 min, 200 µl supernatant was added to 50 µl color reactivator. The mixture was incubated at 37°C for 3 hours and the temperature was made as 0°C. Then 300 µl 65% H_2SO_4 solution was added and the absorbance at 515 nm was measured immediately after mixing[16].

Statistical Analysis

All measurements were done in a blinded fashion on precoded samples. Data analysis was carried out with SPSS 9.01 (SPSS, Inc., USA). Histamine and ascorbic acid levels of matched experimental and control pairs were analysed with the non-parametric, Mann-Whitney U-test. The results were expressed as means with their standard errors. Only two-sided results were used. P values of 0.05 or less were considered significant. The confidence intervals of the related means were also mentioned.

RESULTS

Twenty-eight rats were used in the study and randomized according to irradiation and drug administration. The histamine levels (µg/ g tissue) and ascorbic acid levels (mg/ g tissue) present in the wound tissue are shown in Table 1 and Figure 1. The animals given TBI demonstrated marked increase in

Table 1. Histamine and ascorbic acid content level in wound tissue

GROUPS	PARAMETERS	
	Histamin (µg/g tissue) Mean±SE*	Ascorbic acid (mg/g tissue) Mean±SE*
Nonirradiated		
Group 1	8.29±0.92[a]	35.08±2.62[e]
Group 2	5.97±0.51[b]	25.48±2.19[f]
Irradiated		
Group 3	34.84±3.25[c]	17.02±1.43[g]
Group 4	6.41±0.80[d]	16.75±1.83[h]

*SE- Standard Error of Mean

Group 1: Control, Group 2 : GM-CSF administered, Group 3: Irradiated control group, Group 4: Irradiated and GM-CSF administered group.

95% CI; (a) 6.01 to 10.56; (b) 4.70 to 7.23; (c) 26.89 to 42.79; (d) 4.44 to 8.37; (e) 28.67 to 41.49; (f) 20.09 to 30.85; (g) 13.52 to 20.52; (h) 12.28 to 21.23 (a,b) p=0.048, (a,c) p= 0.002, (e,f) p=0.018, (e,g) p=0.002 (b,d) p=0.655, (c,d) p=0.002 (f,h) p=0.018, (g,h) p=0.949

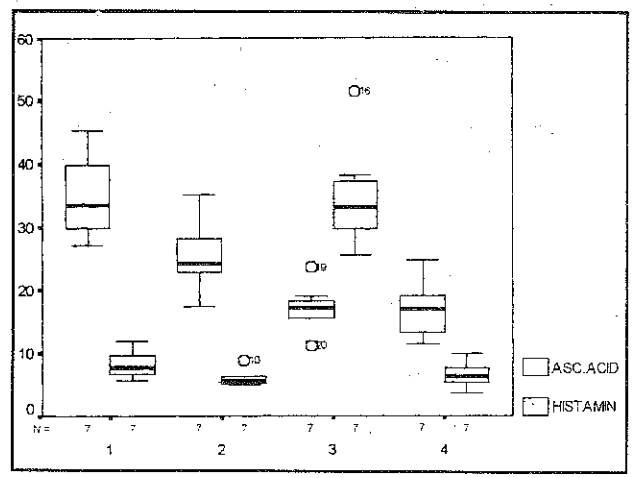

Figure 1. Boxplot diagram of histamine and ascorbic acid content level in skin wound tissue
Group 1: Control, Group 2 : GM-CSF administered, Group 3: Irradiated control group, Group 4: Irradiated and GM-CSF administered group.

the histamine content. Nine days after wounding (11 days after irradiation) histamine content in the irradiated control animals was 420% of that of the non-irradiated control animals (groups 1 and 3) (95% CI-group 1, 6.01 to 10.26 and group 3, 26.89 to 42.89) (p=0.002). Irradiation resulted in an increase in the mean histamine content. The mean values of histamine content were 34.84 (95% confidence interval (CI), 26.89 to 42.79) and 6.41 µg/ g (95% CI, 4.44 to 8.37) for the groups 3 and 4, respectively. According to these results; in animals given TBI, a single topical dose of GM-CSF (15 µg) applied two days after irradiation decreased histamine content of the tissue to 18% of the matched irradiated control incisions on postwounding day 9 (groups 3 and 4) (p=0.002). In the matched GM-CSF administered, irradiated and non-irradiated groups (groups 2 and 4) no difference was found in the histamine content levels (p=0.655). No significant difference was found due to histamine content according to confidence intervals in the non-irradiated control and drug administered groups (groups 1 and 2) (95% CI- group 1, 6.01 to 10.26 and group 2, 4.70 to 7.23; respectively). (p=0.048). Although the difference seems statistically significant, the CIs indicate that the degree of confidence is low for these means.

By irradiation ascorbic acid content of the skin tissue was found decreased to 48% in the non-irradiated control group compared to the matched irradiated control group (groups 1 and 3) (95% CI-group 1, 28.67 to 41.49 and group 3, 13.52 to 20.52). This difference was found statistically significant (p=0.002). In the non-irradiated control and drug administered groups (groups 1 and 2), the mean values of the ascorbic acid content were 35.08 (95% CI, 28.67 to 41.49) and 25.48 mg/ g (95% CI, 20.09 to 30.85); respectively (p=0.018). Although the difference seems statistically significant, the CIs indicate that the degree of confidence is low for these means. The ascorbic acid content was found to be lower in irradiated, drug administered group than the non-irradiated, drug administered groups (groups 2 and 4) (p=0.018).

DISCUSSION

Pre- or post-operative radiotherapy or chemoradiotherapy is frequently used in current oncology protocols. The aim of the radiation oncologist is uncomplicated loco-regional control of cancer by radiotherapy. However, radiation may interrupt normal wound healing mechanism. Changes in vasculature, effects on fibroblasts and varying levels of regulatory growth factors result in the potential for altered wound healing whether radiation is given before or after surgery[17]. These results demonstrate that a single application of GM-CSF diminishes the histamine content of the skin wound, which was increased by irradiation, and has no effect on the ascorbic acid content of the skin wound in rats receiving gamma irradiation at a dose equivalent to that used in clinical applications.

A number of growth factors and cytokines have been described which regulate wound repair, wound cell migration and collagen synthesis. GM-CSF has been the only cytokine with proven in vitro and in vivo activity on the proliferation of keratinocytes[18]. Locally applied GM-CSF has been shown to accelerate the healing strength of wounds in a number of animal model systems, including immuncompromised animals and infected wounds[18]. The breaking strength of the scar tissue when measured by tensiometry was 42% stronger at day 9 in rats given GM-CSF than in controls[5]. In humans, locally applied GM-CSF accelerates the healing of chronic leg ulcers, Kaposi's sarcoma, burn wound and skin graft[19]. GM-CSF administration was also found effective in the acceleration of skin wound healing by histological examination[20].

Histamine was shown to enhance fibroblast migration and proliferation in an in vitro wound model[7]. By the inhibition of histamine synthesis, breaking strength and hydroxyproline content of the granulation tissue were found decreased and the period of epithelization was delayed[21]. Norrby et al.[22] demonstrated that only low, almost physiological concentration of histamine are mitogenic to dense fibroblast culture. It was also reported that exogenous histamine has prohealing action only when endogenous histamine level is suboptimal[23]. In the present study, the total body irradiation caused an increase in his-

tamine content of wound tissue. These findings indicate that skin mast cells are activated by irradiation. In our study, GM-CSF administration was found to decrease histamine content of wound tissue in the irradiated animals in the current study. This decrease suggests that GM-CSF may modulate mast cell activity and preserves histamine concentration in physiological levels in the irradiated wound environment. There are some data which indicate that histamine at low doses stimulate and at high doses inhibit collagen biosynthesis[9].

In the literature, it was suggested that the wound healing process was accompanied by the oxidation of ascorbic acid in the wound area[24]. Ascorbic acid is also known to be a powerful anti-oxidant. It has been postulated that ascorbic acid is a radioprotectant as a result of free radical scavenging. Ascorbic acid solution offers the potential for delivery of pharmacologic levels of ascorbic acid to the skin to improve collagen synthesis and antioxidant functions[11]. In the current study, although the drug was found to have no effect on the ascorbic acid content in the statistical analysis, in fact it did not cause any decrease of the ascorbic acid content which was decreased by irradiation. This negative conclusion may be a result of insufficient sample size or drug dosage. To test the effect of GM-CSF at a single dosage and time might have been a study limitation.

Although GM-CSF usage is popular in wound healing models and in prevention of mucositis due to chemotherapy or radiotherapy, it is very expensive. Thus further more detailed investigations are required to determine the mechanisms involved. GM-CSF has been known to improve wound healing, but its effectiveness on the irradiated skin wound has not been well known . This study aims to determine the effect of GM-CSF on other important factors taking part in wound healing. To study the antioxidant enzymes, such as superoxide dismutase, histamine synthetase, etc. may provide other interesting information as what is happening at the cellular level. It is also interesting that GM-CSF functions effectively in the rat and is not destroyed as a foreign protein. It is known that the source of GM-CSF (human or murine) is independent of the outcome so that GM-CSF may be non-specific among species with respect to wound healing[18].

TBI is known to cause a pronounced decrease in antioxidant capacity and an excessive increase in oxidant stress. In our other study, topical administration of GM-CSF was found to decrease lipid peroxidation and to increase the reduced glutathione content of the irradiated skin wound[25]. It is widely accepted that macrophages are the critical inflammatory cells required for wound healing and macrophage release of cytokines into wounds is a rate-limiting factor in the healing process, so any agent that attracts or activates macrophages may exhibit a positive effect on wound healing as an activator of macrophages[5]. So GM-CSF may be useful in irradiated subjects, but their advantage must be weighed carefully against their adverse effects. One of the limitations of the current study is the fixed dose and administration day of GM-CSF. Although different dose and administration timing might provide interesting information, a fixed dose and administration day, still used in routine practice, was chosen as no financial support could be obtained for the study.

This study shows that the conventional dose of GM-CSF reduces the histamine content elevated by radiotherapy in the irradiated rats. The current findings are thought to lead to further investigations about the role of GM-CSF on radiation-induced normal tissue toxicity to determine the mechanisms involved and the optimum dosage schedule.

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