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ORIGINAL ARTICLE

A new concept in the treatment of extravasation injury: controlled drug delivery systems

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Summary

Purpose: *To investigate the effectiveness of the intralesionally injected controlled granulocyte-monocyte colony stimulating factor (GM-CSF) releasing system in widening refractory extravasation wounds.*

Methods: *The determination of in vitro GM-CSF release from chitosan gel was the first, and in vivo effect of the molecule was the second step of the study. Thirty-five Wistar-Albino rats were randomly divided into 5 groups: 1) control group (adriamycin group) (n=7); 2) adriamycin + normal saline group (n=7); 3) adriamycin + chitosan group (n=7); 4) adriamycin + 1 μg/mL GM-CSF-loaded chitosan group (n=7); and 5) adriamycin + 10 μg/mL GM-CSF loaded chitosan group (n=7). The wound area was measured macroscopically and histological examination was carried out for wound healing and tissue response to the polymer.*

Results: *The best healing process was observed with the controlled released GM-CSF groups (groups 4 and 5). The 1 μg/mL GM-CSF loaded group showed superior wound healing than that of 10 μg/mL GM-CSF loaded gels. This result was correlated with the in vitro study which also showed increased drug release in the 1 μg/mL GM-CSF loaded group than the 10 μg/mL GMC-SF loaded gels.*

Conclusion: *This study suggests that GM-CSF, applied with controlled drug delivery system, can supply dynamic treatment options with long-lasting activity in single-dose administration.*

Key words: adriamycin, chemotherapy, chitosan, controlled release, extravasation

Introduction

Extravasation injury is defined as tissue damage caused by leakage of different kinds of intravenous medication solutions to the interstitial space of a vein's surrounding tissue. In the literature the incidence of extravasation injury in cancer patients has been reported from 0.1 to 6% [1]. It may reach 11-58% in children [1,2]. The most common pharmacologic agents causing extravasation injury are osmotic active chemicals such as total parenteral nutrition, cationic solutions like potassium [K^+], calcium [Ca^{++}] and cytotoxic drugs [3]. Among cytotoxic agents adriamycin (doxorubicin hydrochloride) is the chemotherapeutic causing the most serious extravasation injury [4,5].

Clinical manifestation of adriamycin extravasation is characterized by immediate pain, erythema and swelling at the extravasation site [6,7]. Ulceration may appear after some days or a week and continues worsening for months probably because of difficulties of drug metabolism and long duration of action of the agent. Adriamycin binds to tissue DNA after immediate injury and is released continually from dying to healthy cells, resulting in a slow increase of ulcer size over the course of time. Progressive ulceration may extend deep to the underlying structures such as tendons and bones resulting in loss of joint functions [8]. The extravasated drug has been shown to remain in tissues for 5 months after leakage, which means extensive tissue destruction continuing in the late phase of the injury [3].

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The main role of GM-CSF is stimulation and proliferation of the hematopoietic progenitor cells in the myeloid lineage into macrophages, neutrophils and eosinophils. The agent also acts as an inducer for endothelial cells on migration and proliferation related to angiogenesis [9]. Several reports have demonstrated that locally applied GM-CSF promotes wound healing [10-14].

Due to its good biocompatibility and low toxicity profile, chitosan has been widely used in pharmaceutical research and in industry as a carrier for drug delivery [15]. Because chitosan's amino and hydroxyl groups are easy to be modified, it can produce other derivatives. Chitosan can be used as carrier in controlled slow released drug systems in the form of microcapsule, porous block or gelatin.

We hypothesized that long-lasting activity of GM-CSF by controlled slow-released hydrogel from chitosan against progressive ulceration would increase differentiation of the hematopoietic progenitor cells and interact with other cytokines over a long period of time. Then all these effects would enhance wound healing.

Methods

The experiment was designed in two stages: a) the determination of *in vitro* GM-CSF release from chitosan gel; and 2) *in vivo* effect of GM-CSF loaded chitosan on extravasation injury. Thirty-five Wistar-Albino rats, weighing 250-300 g, were used. Anesthesia was initiated with intramuscular injection of a cocktail of ketamine hydrochloride 90 mg/kg and xylazine 10 mg/kg, and – if needed – supplementary doses were administered during the procedure. All animal experiments were in full agreement with the Turkish law 6343/2, Veterinary Medicine Deontology Regulation 6.7.26, and with the Helsinki Declaration of animal rights. Hacettepe University Animal Care Ethical Review Committee approved the design and procedures of the study (#2005/12-8).

All rats were injected with 1 mg adriamycin intradermally in 0.5 ml saline [8]. In the first 2 hours after injection the rats were randomly divided into 5 groups: 1) control group (adriamycin group) (n=7); 2) adriamycin + normal saline group (n=7); 3) adriamycin + chitosan group (n=7); 4) adriamycin + 1 μg GM-CSF loaded chitosan group (n=7); and 5) adriamycin + 10 μg GM-CSF loaded chitosan group (n=7). 0.1 ml of saline, chitosan gel or chitosan gel with different concentrations of GM-CSF was injected to extravasation sites of the animals to standardize the dilution of the injected adriamycin. Two of the animals developed infection (adriamycin and adriamycin+saline groups) and one of them died because of anesthesia complication, so the experiment was completed with 32 rats. The animals were kept in a controlled environment for 5 days with appropriate analgesia.

Preparation of GM-CSF loaded chitosan gels

Chitosan H (MW: 1400 kDa, deacetylation degree 80%) was purchased from MGF Co. Ltd, Japan. Chitosan 2% and 1.5% was dispersed in 0.1 M acetate buffer (pH 4.5) and was allowed to be gelled overnight at room temperature. rhGM-CSF 10 μg/ml and 1 μg/ml was incorporated to chitosan gels and which were then sterilized by 25 kGy of gamma-irradiation at 4° C before being applied to rats for *in vivo* studies. Four formulations were used in the experiments to assess the release kinetics of the molecule:

A1: 10 μg/mL rhGM-CSF loaded to 1.5% chitosan gel
A2: 10 μg/mL rhGM-CSF loaded to 2% chitosan gel
B1: 1 μg/mL rhGM-CSF loaded to 1.5% chitosan gel
B2: 1 μg/mL rhGM-CSF loaded to 2% chitosan gel
(Table 1).

In vitro release of GM-CSF from gels

200 μL of GM-CSF containing chitosan gels were placed in the diffusion cells. *The in vitro* release of GM-CSF from chitosan gels was measured through a microporous cellulose membrane which was mounted on the cells' diffusion. These cells were placed in 15 mL vials containing 10 ml release medium (PBS, pH 7.4 with 1 mM PMSF) and agitated on a water bath at 4° C. At predetermined time intervals (0.25, 0.5, 1, 2, 4, 8, 24 and 48 hours) 50 μL samples were taken from the diffusion medium and protein amounts were analyzed by ELISA method. The equation for the standard curve was y=0.004x+0.069. Triplicate samples were measured for each time point.

The wound area was measured macroscopically before samples were obtained for morphological analysis. The area of necrosis was transferred to a transparent malleable paper, then the area marked on the transparent paper was measured using scaled graph paper at periodic intervals. Then, the obtained specimens were placed in 10% phosphate buffered formalin (pH 7.0) at

Table 1. Formulations used in this study

Formulation	*Chitosan gel concentration (%)*	*GM-CSF concentration (μg/ml)*
A1	1.5	10
A2	2	10
B1	1.5	1
B2	2	1

room temperature for fixation. Samples were rinsed in buffer before dehydration in a graded series of ethanol for embedding in paraffin. Five micrometer thick serial sections were cut from the open wound site with a sliding microtome (Microm, HM 360, Waldorf, Germany). Haematoxylin & eosin and Goldner's Masson Trichrome stained sections were evaluated for wound healing and tissue response to the polymer. Histological observations were graded in detail according to a histological wound healing scale [16] by two independent investigators using Leica DMR microscope (Leica, Wetzlar, Germany). The images were captured by Leica DC500 digital camera (Germany). The 5-category histological scoring system is summarized in Table 2.

Statistical analysis

Statistical analysis was performed using the SPSS for Windows Release 11.5. Histological scores and macroscopic measurements were analyzed using non parametric tests (Kruskal Wallis and its post-hoc tests) to assess statistical significance between experimental groups. Spearman's test was used to assess correlations between continuous variables, macroscopic measurements and histological scores. Probability value (p) of less than 0.05 was considered as significant.

Results

According to *in vitro* experiments, 1 μg/mL drug loaded gels showed better release kinetics than the 10 μg/mL loaded gels. As the loaded protein amount increased, the released amounts from the chitosan gels decreased. The *in vivo* study demonstrated that controlled GM-CSF releasing groups exhibited superior wound healing than the other groups. The best healing process was observed with 1 μg/mL GM-CSF loaded group which was correlated with the *in vitro* study.

In vitro release profiles of GM-CSF from chitosan gels

The amount of GM-CSF released from the chitosan gel was found to be dependent on the concentration of the protein loaded to gel. As the loaded protein amount increased, the released amounts from the chitosan gels decreased. The released amounts of the GM-CSF from chitosan gels can be seen in Figure 1. After 48 hours, the release from the chitosan gels consisting of 10 μg/ml protein reached 8.5%, and the release from the chitosan gels consisting of 1 μg/ml protein reached 42%. Chitosan gel concentrations did not show an effect on the release profiles (Figure 1). Release kinetics of B1 and B2 formulations were fit with Higuchi square root of time relationship (B1 formulation r2 =0.980, B2 formulation r2=0.971).

The macroscopic wound area was greatest in the adriamycin control group (group 1) compared to all other groups (groups 2-6) ($p < 0.05$; Figure 2A). The wound area was significantly larger in group 2 (adriamycin+saline; Figure 2C) compared to group 4 (adriamycin+ chitosan+GM-CSF 1 μg) (Figure 2B; $p < 0.05$). The wound area was significantly larger in group 3 (adriamycin+ chitosan) compared to group 4 (adriamycin+ chitosan+ GMC-SF 1 μg) and 5 (adriamycin+ chitosan+GM-CSF 10 μg) (Figures 2B, 3A-B, 4) ($p < 0.05$).

Histological scores for reepithelization, number of recruited inflammatory cells and macroscopic wound

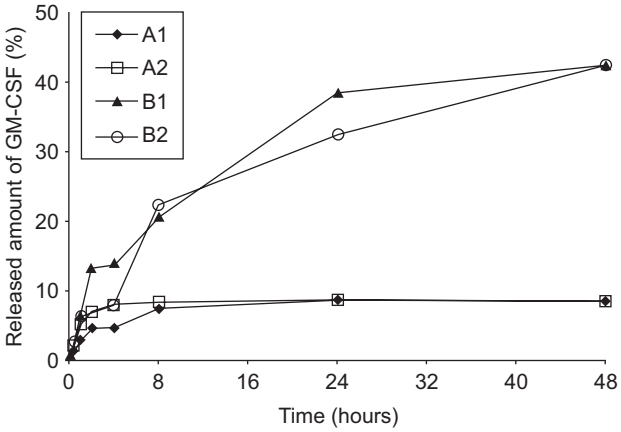

Figure 1. Release profiles of GM-CSF from chitosan gels. For formulations A1, A2, B1, B2 see text.

Table 2. Five-category histological wound healing scoring system

	0	*1*	*2*	*3*	*4*
Reepithelization	Not found	Thin	Moderate	Full thickness	
Granulomatous tissue formation	Not found	Less than 30%	More than 31%, less than 60%	More than 61%, less than 99%	Complete granulomatous tissue formation
Number of inflammatory cells	Normal	Slight	Moderate	Severe	
Number of fibroblasts	Normal	Slight	Moderate	Severe	
Number of neovasculature	Normal	Slight	Moderate	Severe	

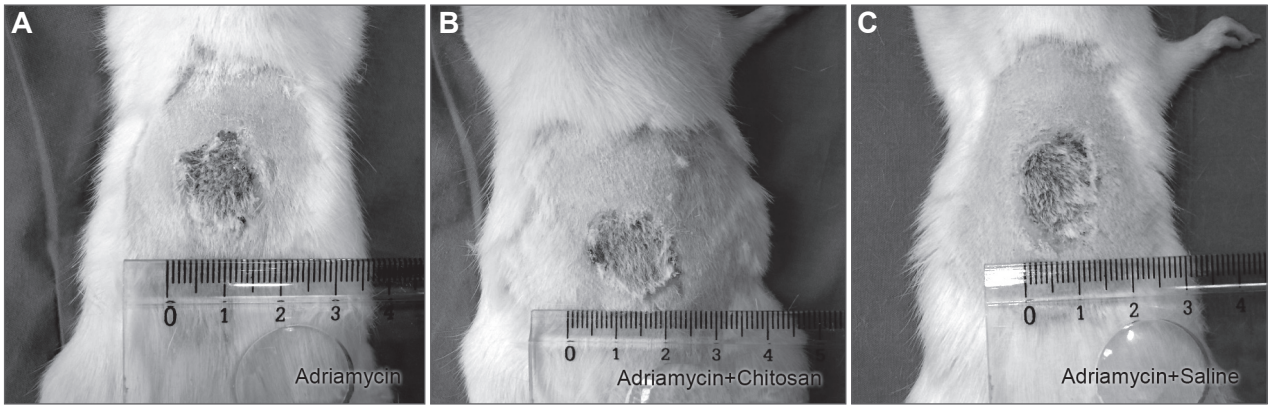

Figure 2. A: Mean macroscopic wound area was largest in the adriamycin group (group 1). The wound area was significantly larger in group 2 (adriamycin+saline) (**C**) and in group 3 (adriamycin+chitosan) (**B**) compared to controlled GM-CSF released groups.

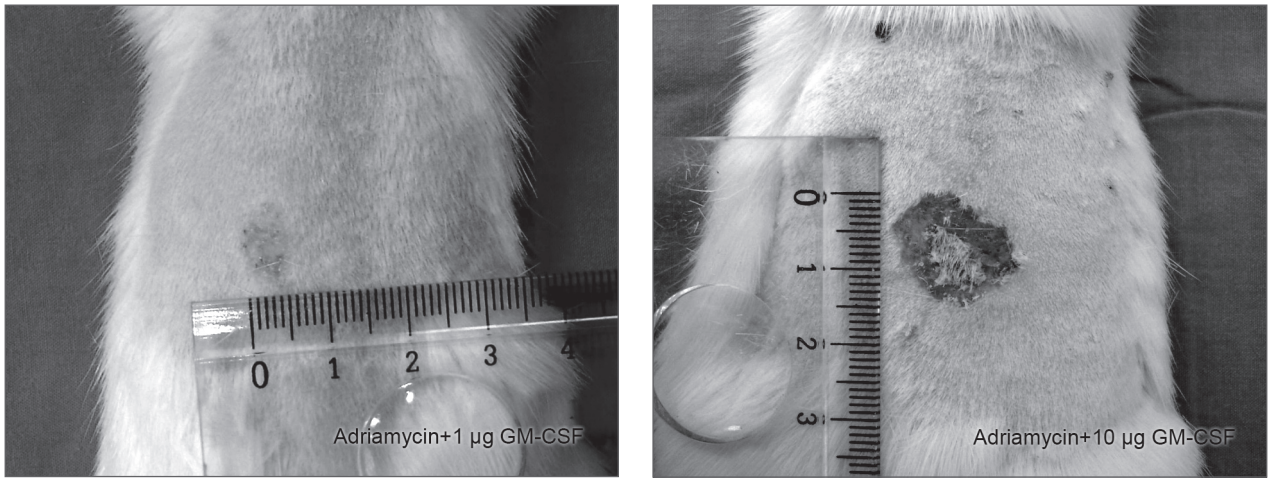

Figure 3. Controlled GM-CSF released groups show better wound healing and the 1 µg GM-CSF group displays superior epithelization than the 10 µg GM-CSF group.

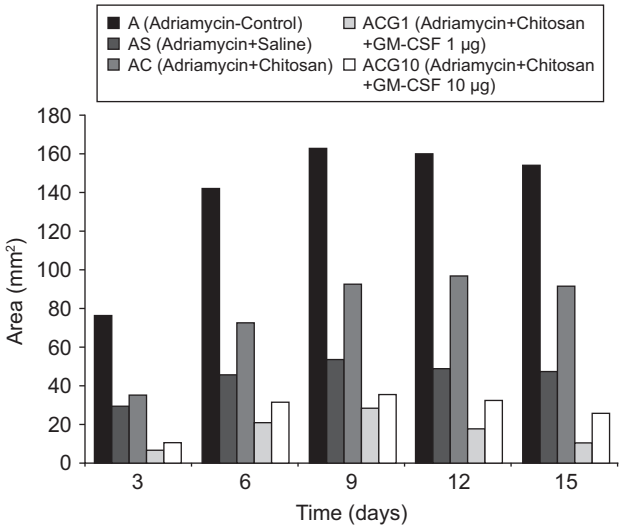

Figure 4. Measured wound areas at periodic intervals can be seen. The best healing process was observed in the controlled released GM-CSF groups. The 1 µg/mL GM-CSF loaded group showed superior wound healing than the 10 µg/mL GM-CSF loaded group.

area measurements exhibited statistically significant differences among groups ($p < 0.05$; Table 3). Histological reepithelization scores were significantly higher in the GM-CSF applied groups 4 (adriamycin+chitosan+GM-CSF 1 µg) and 5 (adriamycin+chitosan+GM-CSF 10 µg) compared with groups 1, 2 and 3 (adriamycin, adriamycin+saline and adriamycin+chitosan, respectively) ($p < 0.05$).

The number of inflammatory cells was significantly higher in the control groups (adriamycin only and adriamycin+saline) when compared to chitosan gel and GM-CSF loaded chitosan gel groups ($p < 0.05$). Group 5 (adriamycin+chitosan+GM-CSF 10 µg) displayed significantly lower scores in relation to the number of recruited inflammatory cells comparing to group 3 (adriamycin+chitosan) ($p < 0.05$).

Macroscopic wound area measurements were in strong negative correlation with histological reepithelization scores ($r = -0.839$, $p < 0.05$), and in strong positive cor-

Table 3. Macroscopic and histological scores (raw data)

Groups/ Parameters (mm2)	*Macroscopic wound area*	*Reepithelization*	*Granulomatous tissue formation*	*Number of inflammatory cells*	*Number of fibroblasts*	*Number of neovasculature*
A (Adriamycin-Control)						
1	184	1	3	3	2	2
2	140	1	3	3	2	2
3	182	1	3	3	2	2
4	83	2	2	3	2	3
5	156	1	3	3	2	2
6	172	1	3	3	2	2
AS (Adriamycin+Saline)						
1	119	1	3	3	2	2
2	78	1	2	3	2	3
3	37	2	3	3	2	3
4	17	2	3	2	3	2
5	18	2	3	3	2	2
6	8	2	3	2	3	3
AC (Adriamycin+Chitosan)						
1	172	1	3	3	2	2
2	137	1	3	2	2	2
3	10	2	3	2	3	3
4	13	2	3	2	2	3
5	133	2	3	3	2	2
6	133	1	3	3	2	2
7	36	2	2	2	2	2
ACG1(Adriamycin+Chitosan +GM-C. SF 1 μg)						
1	2	3	2	1	2	3
2	4	3	2	2	2	3
3	6	3	3	2	2	3
4	2	3	3	2	3	2
5	12	2	3	2	2	2
6	29	2	3	3	2	2
7	6	2	3	2	2	3
ACG10 (Adriamycin+Chitosan +GM-CSF 10 μg)						
1	23	2	3	2	2	2
2	19	3	3	2	2	2
3	17	3	3	2	2	2
4	72	2	2	2	2	3
5	9	2	3	1	3	2
6	7	3	3	1	3	3

relation with the number of inflammatory cells (r=0.779; $p < 0.05$). Macroscopic wound area measurements were moderately negatively correlated with both the number of fibroblastic cells (r=-0.477) and the number of the neovasculature in the wound area during healing (r=-0.486; $p<0.05$ each). All chitosan applied samples of groups 3, 4 and 5 presented minor tissue reaction to chitosan, which was used as carrier. The small degrading polymer particles were occasionally observed to be surrounded by few mononuclear phagocytic and fibroblastic cells.

The best healing process was observed with GM-CSF applied groups (groups 4 and 5) in which the wound area was significantly narrowed by favorable reepithelization rate and granulomatous tissue formation characterized by recruitment of inflammatory cells leading the neovascularization and collagen fibril reorganization by myofibroblasts (Figures 5, 6). Recruitment and the increase of inflammatory cells caused by extravasation and edema subsided earliest at the bottom of the wounds in the chitosan+GM-CSF applied groups. Subsequently, fibroblasts and the newly formed capillaries proliferated and prearranged more remarkably for the recovery of dermal connective tissue at the injury side in the chitosan+1 and 10 μg GM-CSF applied groups. Interestingly, 1 μg GM-CSF+chitosan gave favorable results compared to 10 μg. On the other hand, since the damage involved all skin layers including both the epidermis and the whole dermal connective tissue, wound healed with different degrees of scar tissue formation in the dermis in both control and GM-CSF applied groups.

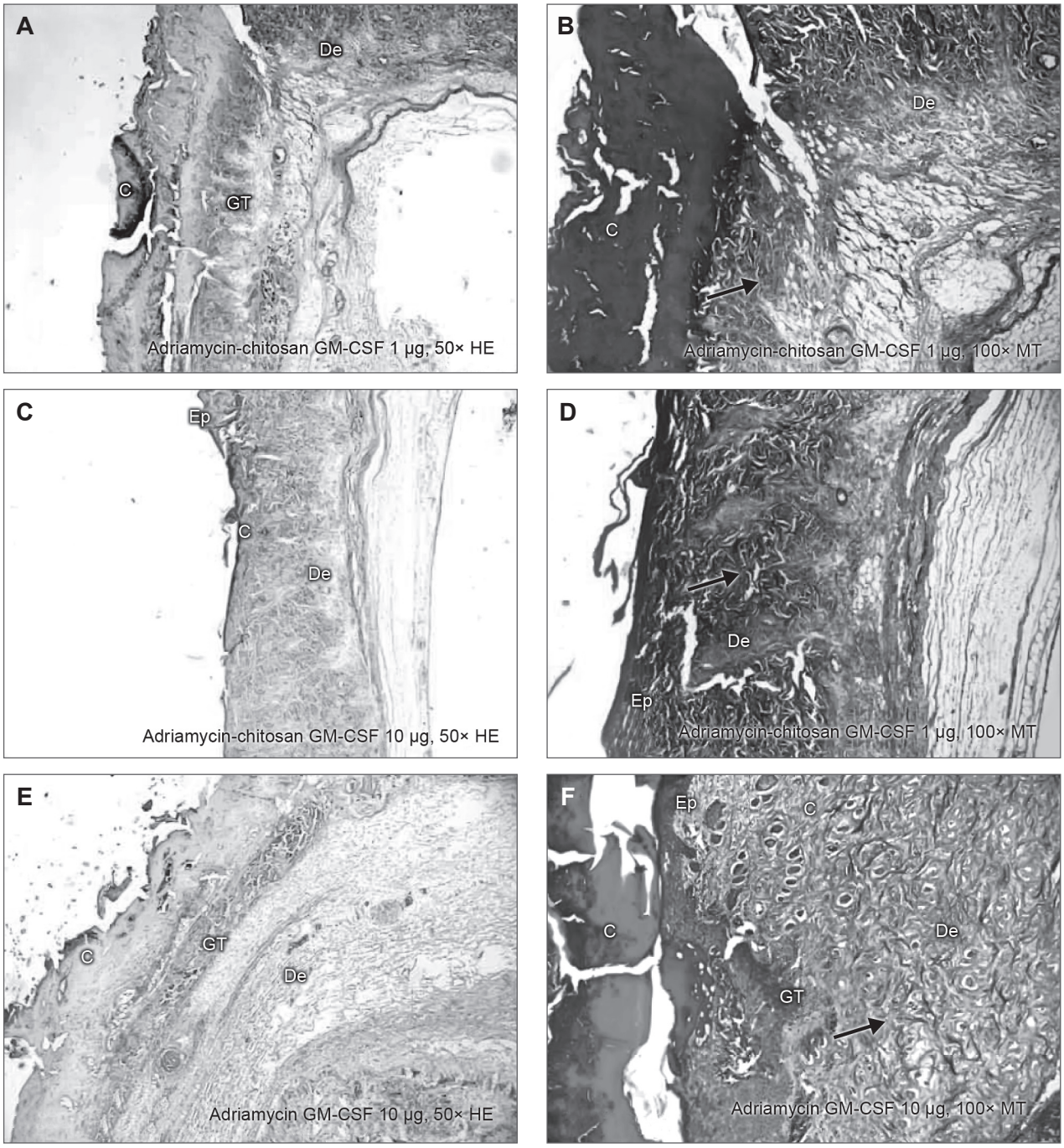

Figure 5. A-F show wound area consisting of loss of epidermis, a loosely rearranged granulomatous tissue containing adiposities and different degrees of dermal cicatrices adjacent to the granulation tissue; **C** and **D** are from the edge of a big wound. So the dermis looks more organized into dense fibrous connective tissue than that of other micrographs. Note that the narrowed wound area is limited by thin epidermal lining in **E** and **F**. Ep: epidermis, GT: granulomatous tissue, De: dermis, C: crust over wound, HE: haematoxylin-eosin, MT: Masson's trichrome. Arrows show collagen fibres.

Discussion

The present study is the first to pursue the effectiveness of the intralesionally injected controlled releasing system in widening refractory wounds. Here we demonstrated the contribution of the single-dose controlled-releasing GM-CSF from chitosan gel on wound healing of adriamycin extravasation injury. Best healing process was observed in the controlled released GM-CSF applied groups (groups 4 and 5) in which the wound area was significantly narrowed. The 1 µg/mL GM-CSF loaded group (group 4) showed superior wound healing than group 5 (10 µg/mL GMC-SF loaded gels). This result was correlated with the in vitro study which also

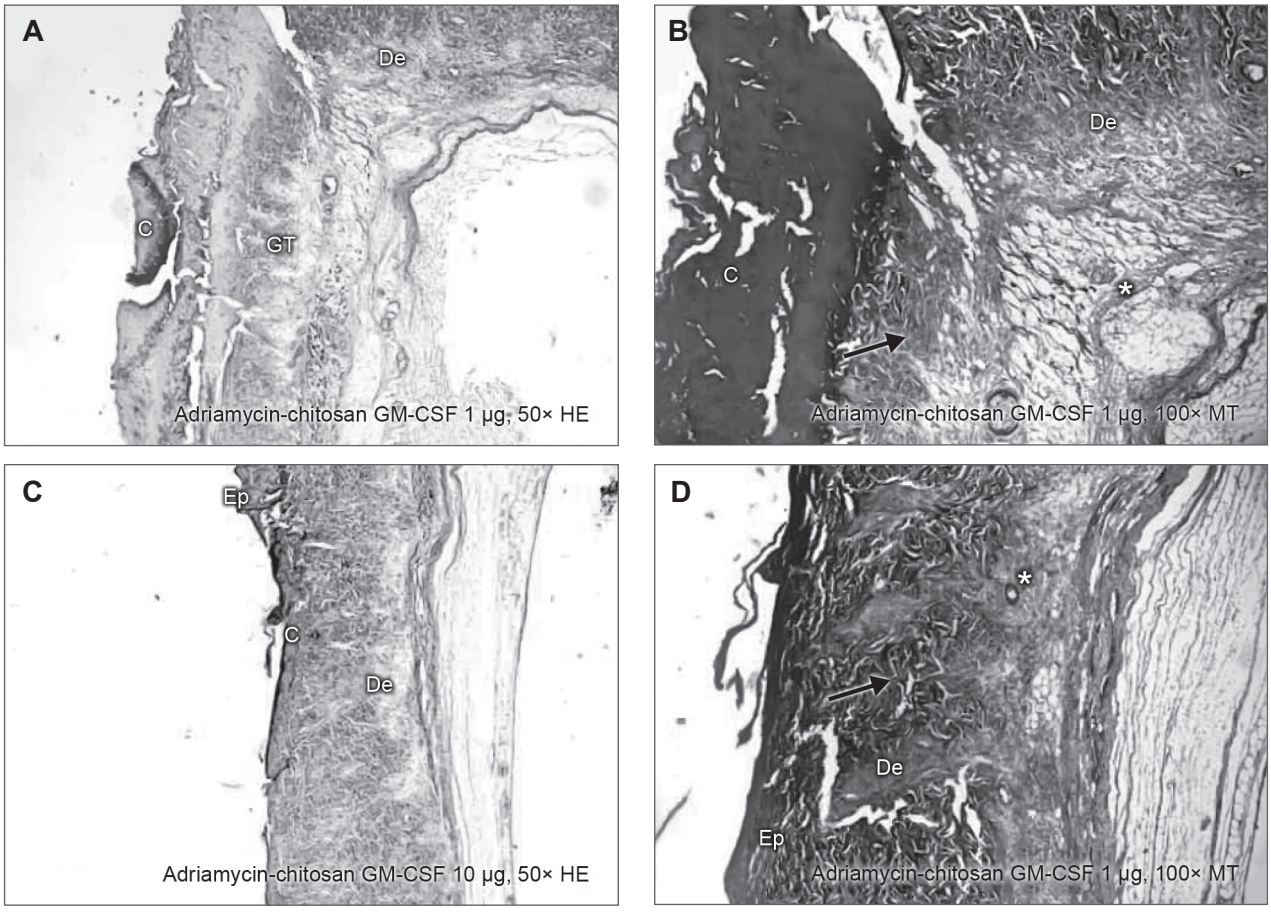

Figure 6. A-D show narrowed wound area belonging to GM-CSF groups samples. All of the micrographs were selected from samples in which the wound area was deepest. Granulomatous tissue is organized into fibrous connective tissue containing densely packed collagen fibres. Note that the wound area is markedly narrowed and recovered in **A** to **D**. Ep: epidermis, GT: granulomatous tissue, De: dermis, C: crust over wound, HE: haematoxylin-eosin, MT: Masson's trichrome. Arrows show collagen fibers, asterisk: blood vessels.

showed increased drug release in the 1 µg/mL GM-CSF loaded group than the 10 µg/mL GM-CSF loaded gels.

There are several limitations in our study. First, the small sample size which was arranged by the ethics committee limited our statistical power to non-parametric tests. Second, gene expression on biopsy specimens was not carried out to observe temporal events of wound healing. Finally, due to the limited sample size, slow released amount of GM-CSF in the *in vivo* study was not detected. Despite these limitations, the results of the study were not affected negatively.

Serious extravasation injuries have become more common after more frequent use of chemotherapeutics in medical practice. The incidence of extravasation injury was reported to be 0.1-6% after cytotoxic drug administrations [4,17,18]. Patients undergoing chemotherapy have a 4.7% risk for getting extravasation injury [19]. The incidence of extravasation was reported to be as low as 0.01% at M.D. Anderson Cancer Center, as if this was a rare event [20]. The incidence is closely related with the quality of patient care of the institution, therefore this figure cannot be taken as representative of the whole problem.

The treatment of extravasation injury can be classified as non pharmacologic, pharmacologic and surgical. Aspirations of the extravasated fluid, irrigation of the affected area, cold or hot compresses are some of the non pharmacologic techniques. Pharmacologic agents used for extravasation injury are corticosteroids [21,22], beta adrenergic agents [23], dimethyl sulfoxide [24], sodium bicarbonate [25], diphenhydramine hydrochloride [26], hyaluronidase [27], acetylcysteine sodium [28], cimetidine, and heparin sodium [29]. Each of these agents has different mechanism of action to cope with extravasation injury. Some have vasoconstrictive effect to prevent spread of the extravasated agent. Others prevent the local inflammatory response, which may increase the tissue destruction. Some work as chelating agent, especially for the toxic free radicals. Surgical management of these injuries mainly consists of debridement and skin grafting of the involved areas and flap repairs on occasions with large tissue necrosis [10].

Since these treatment modalities have different mechanisms of tissue destruction and some cause tissue destruction with an unknown mechanism, it is hard to apply a standard treatment for all cases. Therefore, prevention of the extravasation is still the best way of treatment as both surgical and non surgical therapies are far from being satisfactory, and thus more effective methods need to be developed.

GM-CSF stimulates granulocyte/macrophage and eosinophil colony formation *in vitro* and is a potent activator of neutrophils and eosinophils. It increases neutrophil phagocytic activity, leading to the elimination of the doxorubicin-DNA complex released from the dead cells on the ulcer base before it is taken up by the surrounding viable cells at the extravasation site. Beside leukocytes, GM-CSF has a considerable influence on monocyte accumulation and fibroblast and keratinocyte proliferation; it also induces angiogenesis and interacts with several other cytokines, any or all of which may contribute towards wound healing [30]. GM-CSF appears to be a multipotent wound healing cytokine in skin, stimulates the growth of all cellular components *in vitro* that form the structural basis of human skin, i.e., keratinocytes, fibroblasts and endothelial cells [31,32]. GM-CSF is shown to induce activation and differentiation of endothelial cells, including proliferation and migration that relate to angiogenesis [33]. It was also shown to induce contraction of the wound [34]. Since breakdown of vascular integrity at the extravasation site and reduced contraction of wound have particular importance in adriamycin extravasation injury, GM-CSF must have beneficial effects by reversing these processes.

The contribution of local single dose or consecutive injections of GM-CSF on adriamycin extravasation has been studied and discussed before [14,35], but the long duration of the destructive function of adriamycin up to months necessitates lengthening of the treatment period rather than single application of any agent. De Ugarte et al. reported treatment of a chronic wound with GM-CSF applied by way of an infusion pump in a case with neutropenia and phagocytic dysfunction due to glycogen storage disease [36]. However, the external localization of the pump, difficulties in wound cleaning and dressing with the pump and the necessity of repeated drug injections into the pump system would significantly increase the risk of infection and make this system inappropriate for use in clinical practice. There has been no investigation about GM-CSF release with the help of continued controlled drug delivering systems.

The drug delivery concept is a rapidly evolving area of pharmaceutical biotechnology. Bioactive substances such as drugs or hormones have been delivered more effectively at localized areas or specific target organs of the body with the widespread use of biodegradable, biocompatible polymeric materials. Substances used as drug carriers have to be biocompatible and, if possible, to be also biodegradable. They should be widely available and cheep for large-scale production. They should also be sterilized and have appropriate shelf life. Hydrogel-based technologies for controlled release of proteins have recently become the focus of interest. Hydrogels are stabilized by cross-links by ionic or hydrophobic interactions between the polymeric units that form the "backbone" of the gel. Alginate and chitosan are examples of polymers that can be cross-linked by ionic interactions [37]. In this study, chitosan was preferred to be used as a polymeric carrier system because of its properties of non-toxicity, biocompatibility and having the capacity to bind most of protein-based molecules due to its free cationic charges in its molecular structure.

Known parameters for release kinetics of a protein are the molecular weight of the drug, swellability and degradability of the polymer, solubility of the drug in water and hydrophobic interactions between drug and hydrogen network [38]. Drug release rates depend on the concentration of the drug loaded in polymeric vehicles. Higher drug loading decreases the diffusion rate up to 50% [39]. In this study, after 48 hours, the percent release from 10 μg/mL drug loaded gel (8.5%) was 5-fold lower than the 1 μg/mL drug loaded gels (42%). This behavior can be explained by the decreased solubility of GM-CSF in the gel and blocking effect on the membrane. Release profiles were similar, indicating that the membrane was not a rate limiting factor. The *in vivo* wound healing results with 1 μg/mL drug containing gels were better than the 10 μg/mL gels, correlating with the *in vitro* release results.

Extravasation of adriamycin causes destructive and expanding tissue necrosis for long time after leakage of the agent. This long-lasting toxic activity of the drug should be treated dynamically in that period. This study suggests that the GM-CSF medication, applied with controlled drug delivery system, can supply dynamic treatment options with long-lasting activity with a single dose application. However, prevention of the extravasation is still the best way of treatment. Future research on cancer treatment would bring non toxic chemotherapeutics or anticancer drugs and also prophylactic antinecrotic agent combinations into treatment protocols.

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