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New cytokine dressings. I. Kinetics of the in vitro rhG-CSF, rhGM-CSF, and rhEGF release from the dressings

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Abstract

Wound repair-stimulatory activities of various cytokines and growth factors depend on successful delivery of these factors to the injured sites. Here were present the design and preparation of the new collagen- and polyurethane-based dressings containing the recombinant human cytokines-rhG-CSF, rhGM-CSF or rhEGF. To test the efficacy of the retrieval of the incorporated cytokines, their controlled release from the dressings was carried out over three consecutive days using polyurethane sponge as a collector of the extracts. The maximum quantities of the released rhG-CSF, rhGM-CSF and rhEGF reached approximately 25, 50, and 10%, respectively, of the total cytokine contents of the dressings, as assessed by the specific ELISA tests. These data indicate that collagen- and polyurethane dressings containing rhGM-CSF and rhG/CSF may serve as effective tools for the topical delivery of cytokines to wounded tissues.

Keywords: Cytokine dressings; rhG-CSF; rh-GM-CSF; rhEGF; Delivery systems

1. Introduction

Wound healing is a complex biological process regulated by the functions of much more than ten cytokines and growth factors (Bennett and Schultz, 1993; Roesel and Nanney, 1995; Ono et al., 1995; Abraham and Klagsbrun, 1996; Martin, 1997). These factors are produced by platelets, macrophages/monocytes, lymphocytes, fibroblasts, endothelial cells, and mastocytes recruited to or present at wound sites. After binding to the specific receptors on the surface of various effector cells the cytokines and growth factors modify proliferation, differentiation and many functions of these and other cells responsible for a successful repair of the injured tissues (Bennett and Schultz, 1993; Marinkovich et al., 1993; Dipietro, 1995; Moulin, 1995; Stocum, 1995; Slavin, 1996). This recently accumulated data constitute the rationale for trials of pharmacological regulation of

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the process of wound healing, provided an effective system for the topical delivery of an active, repair-stimulating factor(s) is available.

Of the many cytokines and growth factors tested to date, granulocyte colony-stimulating factor (G-CSF), granulocyte-macrophage colony-stimulating factor (GM-CSF), and epidermal growth factor (EGF) have been shown to effectively stimulate such repair-relevant phenomena as acceleration of epithelialisation, removal of the necrotic tissue debris, and elimination (e.g. by way of phagocytosis) of microorganisms which colonize the wounded site and compromise the healing process (Scarffe, 1991; Kaplan et al., 1992; Gailit and Clark, 1994). No wonder, therefore, that either of these cytokines has been successfully used in the treatment of wounds in both the in vivo experimental models as well as in clinical settings (Brown et al., 1986, Brown et al., 1989; Bussolino et al., 1989; Kaplan et al., 1992; Smith et al., 1994; Marques et al., 1994; Pojda and Strużyna, 1994; Jyung et al., 1994).

Recently, various systems for the topical delivery of cytokines and/or growth factors to the injured tissues have been examined and described. These include direct injection of a stimulatory factor into (DaCosta et al., 1994) or application of the cytokine-soaked gauze (Hui et al., 1995) to the wound, covering the wound with a gel, cream or ointment containing a cytokine or a growth factor (Brown et al., 1989; Puolakkainen et al., 1995; Sheardown et al., 1997), spraying the factor over the wounded site (Wang et al., 1996), preincubation of the skin graft with a cytokine before grafting (Pojda and Strużyna, 1994), and use of the genetically engineered biological bandage containing the culture of the growth hormone-producing cells (Andreatta-van Leynen et al., 1993). Unfortunately, no standardized formulation for the topical delivery of the cytokines to wounds has been established and accepted for use in the clinic. In the present paper we describe the design and preparation of a set of 'cytokine dressings' containing the incorporated rhG-CSF, rhGM-CSF or rhEGF, and present the kinetics of the cytokines' release from therein during the three-day long extraction procedure.

2. Materials and methods

2.1. Cytokines

As the recombinant human granulocyte colony-stimulating factor (rhG-CSF) a commercial product Neupogen® (Hoffman-La Roche) was used; the preparation was diluted to the concentration of 5 μg/ml using the 0.05 M acetic buffer pH 4.0 containing 2% mannitol, 0.05% Tween 80; and 0,2% human serum albumin. As the recombinant human granulocyte-macrophage colony-stimulating factor (rhGM-CSF) a commercial product Leucomax® (Sandoz Pharma) was used; after reconstitution of the lyophilized preparation according to the instruction of the manufacturer, rhGM-CSF was diluted to the concentration of 5 μg/ml using the phosphate-citric buffer pH 6.8 (0.05 M citric acid, 0.05 M disodium phosphate, 2% mannitol, 2% polyethylene glycol 4000, and 0.2% human serum albumin). Lyophilized recombinant human epidermal growth factor (rhEGF) was obtained from Calbiochem; the preparation was reconstituted according to the instructions of the manufacturer with 10 mM acetic acid containing 1 mg/ml human serum albumin. The preparation was diluted to the concentration of 5 μg/ml using 0.1 M phosphate buffer pH 6.5 containing 0.1 M sodium chloride, 2% mannitol, and 0.2% human serum albumin.

2.2. Preparation of the dressings

The following types of the cytokine dressings were designed by us and prepared in sterile conditions using sterile materials and reagents: (i) collagen sponge dressings; small pieces ($1 \times 1 \times 0.4$ cm) of the bovine collagen sponge BIOKOL (Stalmed, Poland) were saturated with 0.1 ml of either the rhG-CSF, rhGM-CSF or rhEGF solutions prepared as described above; (ii) polyurethane sponge dressings; small pieces ($1 \times 1 \times 0.3$ cm) of the polyurethane sponge LIGASANO® (Ligamed Medikal Produkte, Germany) were saturated with 0.1 ml of either the rhG-CSF, rhGM-CSF or rhEGF solutions prepared as described above; (iii) three-layered dressings; prepared according to the previously published

method (Grzybowski et al., 1997). Briefly, pieces of the bovine collagen membrane COLLDRES (Colldres Ltd., Poland) were covered with the Flammazine cream (Solvay Duphar, The Netherlands) containing solutions (as described above) of either G-CSF or GM-CSF. Then, the Flammazine layer was covered with thin collagen sponge BIOKOL (Stalmed, Poland). Next, the dressing was pressed at 37°C and was incubated overnight at 4°C. For all types of the dressings the final concentration of the incorporated cytokine was approx. 500 ng/cm^2. This concetration resulted from the loading level of the cytokine used. After preparation, all types of the dressings were stored at 4°C in the moist chamber until they were used for the extraction procedure (see the following part of the text). Some of the dressings containing rhG-CSF or rhGM-CSF (but not the three-layered dressings) were lyophilized and stored at 4°C until they were used for the extraction of the cytokines.

2.3. Extraction of the cytokines

Extraction of the three cytokines from the dressings was carried out at 37°C in sterile conditions; small pieces ($1 \times 1 \times 0.5$ cm) of the LIGASANO (polyurethane) sponge were used as collectors of the cytokines extracted from the dressings. Briefly, the polyurethane collectors were placed into glass Petri dishes and saturated with 0.3 ml of the respective solution used for the above-described dilution of each cytokine. The cytokine-containing dressings were put on the collectors and incubated for 72 h at 37°C; after each 24-h period the dressings were removed and placed on the fresh polyurethane collector. After completion of each day of the extraction, the collectors were put into tubes poured with 1 ml PBS (phosphate buffered saline, pH 7.4), squeezed and the cytokines were allowed to diffuse from therein for 30 min at 37°C.

2.4. Quantitation of the cytokines in the extracts

For examination of the concentration of rhG-CSF and rhGM-CSF in the extracts, the Amersham diagnostic kits 'BIOTRAK–granulocyte colony-stimulating factor human [(h)G-CSF] ELISA system' and 'BIOTRAK–granulocyte-macrophage colony-stimulating factor human [(h)GM-CSF] ELISA system' (Amersham Life Sciences, UK) were used, respectively. Before examination, the extracts were initially diluted (1:50 for G-CSF and 1:200 for GM-CSF) with the standard dilution buffer included in the kits. For examination of the rhEGF concentrations, the diagnostic 'ChemiKine Human Epidermal Growth Factor (EGF) EIA Kit' (Chemicon International, CA) was used. The rhEGF-containing extracts were initially diluted (1:5) with the PBS-T-A (PBS supplemented with 0.1% Tween 80 and 1% human serum albumin) solution.

2.5. Effect of freeze-drying on the release of the cytokines

Additional experiment was performed for investigation of cytokines release from freeze-drying dressings. The collagen or polyurethane sponge 1×1cm dressings saturated with one of the cytokines used in the study was undertaken to lyophilization procedure. Then, pieces of the freeze-drying dressings were placed into a tube containing 1 ml of the buffer used for the dilution of either G- or GM-CSF. After 24 h of incubation at 37°C the cytokines concentration in the extracts was examined as described in the Section 2.4.

3. Results and discussion

Concentration and rate of recovery of rhGM-CSF in the extracts obtained from different types of the 'wet' (i.e., non-lyophilized) dressings are shown in Fig. 1 and Fig. 2, respectively. As indicated in Fig. 1, most of the cytokine was realeased from all three dressings during the first day of the extraction procedure. In terms of the fraction of the incorporated cytokine, this release amounted to approximately 35% of the total cytokine content in the dressings (Fig. 2). Although the quantities of rhGM-CSF released during the second and third day of the extraction were much smaller than during the first day (Fig. 1), the

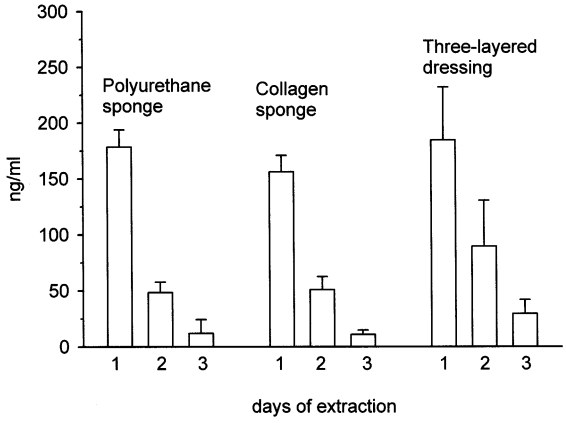

Fig. 1. Release of rhGM-CSF from different types of the cytokine-containing dressings. The bars represent means (± S.D.) obtained from four separate experiments.

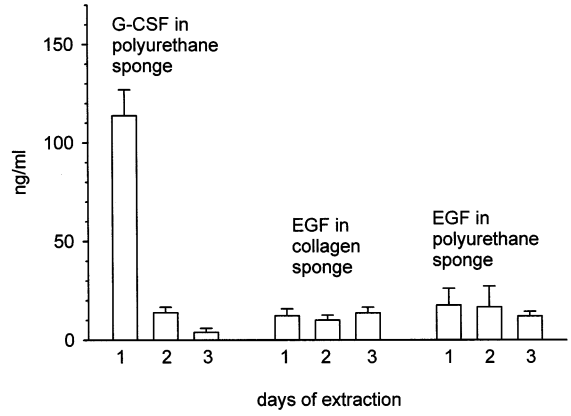

Fig. 3. Release of rhG-CSF and rhEGF from the cytokine-containing dressings. The bars represent means (± S.D.) obtained from four separate experiments.

total, cumulative amounts of the cytokine extracted during the whole period of the procedure approached 50–60% of the cytokine content in the dressings (Fig. 2). Unlike rhGM-CSF, rhG-CSF could be retrieved in substantial amounts only from the polyurethane-based dressings (Fig. 3 and Fig. 4). Despite the fact that similarly to rhGM-CSF this cytokine was released predominantly during the first day of the extraction procedure (Fig. 3), the total quantity of the released rhG-CSF never exceeded 30% of the amount incorporated in the dressings (Fig. 4). The collagen sponge proved to be not a suitable carrier of our preparation of rhG-CSF for which the acidic buffer (pH 4.0) is used as a necessary diluent. Such a low pH impaired the collagen sponge structure leading to the destruction of the dressing. The three-layer collagen dressings was also not suitable as a carrier for rhG-CSF because the cytokine was strongly absorbed and its release was negligible (data not shown).

In contrast to rhGM-CSF and rhG-CSF, retrieval of rhEGF was poor either from collagen or

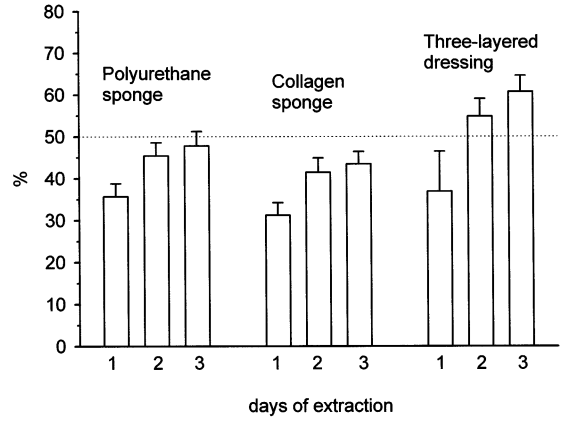

Fig. 2. Efficacy of rhGM-CSF release from different types of the cytokine-containg dressings expressed as total fraction (cumulative data) of the incorporated cytokine. The bars represent means (± S.D.) obtained from four separate experiments.

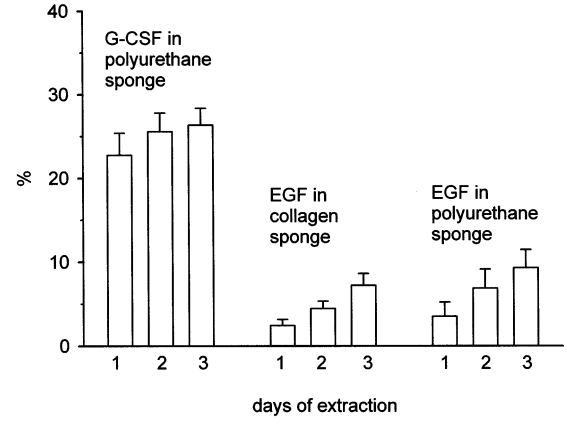

Fig. 4. Efficacy of rhG-CSF and rhEGF releases from the cytokine-containg dressings expressed as total fraction (cumulative data) of the incorporated cytokine. The bars represent means (± S.D.) obtained from four separate experiments.

Table 1
Release of cytokines from two types of freeze-drying dressings (1×1 cm)[a]

Cytokine	Dressing prepared with	
	Collagen sponge	Polyurethane sponge
G-CSF	16.0 ± 4.0 (3%)	98.0 ± 17.0 (20%)
GM-CSF	390.0 ± 136.0 (78%)	499.9 ± 51.3 (100%)
EGF	12.4 ± 3.4 (2.,5%)	12.4 ± 2.9 (2.5%)

[a] The data are expressed in ng and as % of their recovery after 24 h of 1-step exhaustive extraction. Each of the dressings used for the experiment contained 500 ng of the cytokine. The results are presented as means $\pm$ S.D. ($n = 8$).

polyurethane dressings (Fig. 3 and Fig. 4). Indeed, the amounts of the released rhEGF from the both collagen and polyurethane sponges were comparably low during the 3 days of the extraction (Fig. 3), but the maximum cumulative quantity released was always below 10% of the total cytokine content in the dressings (Fig. 4).

The results of the present study clearly indicate that the non-lyophilized, 'wet,' collagen- and polyurethane-based dressings and the non-lyophilized polyurethane–but not the collagen-based dressing can be used as effective carriers and cytokine–delivery systems for the rhGM-CSF and rhG-CSF preparations, respectively.

All the data presented above have documented the release the cytokines from freshly prepared, 'wet' (non freeze-dried) dressings. The three-steps extraction system with using of polyurethane collectors was thought as a model of cytokine release in wound. However, we also employed another, simple one step system of the exhaustive extraction for examination of the cytokines' release from freeze-drying dressings. The comparison of the results presented in Fig. 3 and Fig. 4 and in Table 1 lead us to the conclusion that this one-step extraction produced higher recovery of the cytokines released.

As is shown in Table 1, the release of rhEGF from both types of the lyophilized dressing was very low and did not exceed 3%. The recovery of rhG-CSF from the freeze-drying collagen dressing was similar (average 3%).

Mentioned above disadvantageous influence of the low pH value on the collagen sponge by the buffer used for G-CSF dilution could be minimalized by quick freezing and lyophilization of the dressing. In such a case a time of treatment (at room temperature) of the collagen biomaterial with a buffer of low pH used is very short what resulted in much higher stability of the collagen structure.

The rhG-CSF release from freeze-drying collagen dressing was only 3%. In contrast, the rhGM-CSF release from the freeze-drying dressings was much higher as compared to the three-step procedure and achieved from 78 to 100% depends on the type of the dressing. The low recovery of the CSFs during the three-step extraction may be caused by partial absorption/denaturation of the cytokines in the dressing for 3 days of the experiments. The very low yield (3%) of G-CSF extraction from the lyophilized collagen dressing is not completely clear. Our further experiments with stability of the dressings containing CSFs (Grzybowski et al., Part II, this issue) gave us evidence that G-CSF incorporated into the dressing was stable for 2 months only (after that time the cytokine lost their activity almost completely) as compared to GM-CSF which was entirely stable for 3 months of observation. So, we can conclude that rhG-CSF possess more labile structure of the molecule than rhGM-CSF.

One should emphasise that in the presented study the rate of cytokines release is documented on the basis of the ELISA results. The test can show the antigenic activity of the cytokines only, and the results say nothing about physiological activity of the cytokines extracted. This biological activity of cytokine dressings containing CSFs is presented in the second part of our investigation (Grzybowski et al., Part II, this issue).

To our knowledge, this is the first description of the dressings deliberately designed and prepared for use as tools for topical administration of cytokines to wounds. Since, as indicated in Part II of the present study, the cytokines incorporated in the dressings exhibit potent phagocytosis-related functions of human leukocytes in vitro and antimicrobial activities in vivo (Grzybowski et al., Part II, this issue). We believe that these dressings may constitute a standardized system of the topical delivery of cytokines to wounded tissues.

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