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Immun affinity measurement of recombinant granulocyte colony stimulating factor in patients with chemotherapy-induced neutropenia

Terry M. Phillips

Immunochemistry Laboratory, The George Washington University Medical Center, 413 Ross Hall, 2300 Eye Street, N.W., Washington, D.C. 20037, USA

Abstract

A high-performance immunoaffinity chromatographic technique has been developed for the measurement of recombinant human granulocyte colony stimulating factor in human patients receiving this agent, following neutropenia, arising from cancer chemotherapy. The technique employs a short, biocompatible polymer column packed with minute, antibody-coated glass beads. This system was applied to the analysis of recombinant human granulocyte colony stimulating factor in three different human body fluids. A reasonable degree of correlation was achieved when comparing the immunoaffinity technique to a conventional immunoassay, although the immunoaffinity technique displayed greater specificity.

1. Introduction

Haemopoietic growth factors belong to the family of regulatory peptides known as cytokines and have been shown to regulate the proliferation and differentiation of bone marrow progenitor cells into mature cells [1–3]. Granulocyte colony stimulating factor, a pleiomorphic member of this cytokine family, exerts its effects on the proliferation, differentiation, and activation of haemopoietic cells of the neutrophilic granulocyte lineage [1–5]. *In vitro*, this factor has been shown to stimulate neutrophilic colony formation from bone marrow progenitor cells and *in vivo* to prolong the lifetime, as well as, enhance activity of mature neutrophils [6,7]. Circulating levels of granulocyte colony stimulating factor have been reported as being elevated during infections [8,9], thus indicating a role for this cytokine in host defence mechanisms, especially the activation of neutrophils.

A serious side-effect associated with cancer chemotherapy is the induction of neutropenia, a condition in which the therapy causes a profound decrease in the number of circulating neutrophils, leading to an increased frequency or prolonged duration of infections. Recently, the use of haemopoietic growth factors, especially the granulocyte colony stimulating factor, has become an important issue in cancer chemotherapy both as an agent used to treat chemotherapy-induced neutropenia and as an adjunct treatment modality for haematological cancers [10–12]. Infusion with recombinant human granulocyte colony stimulating factor (rhG-CSF) has been reported as being well tolerated in humans with only minor side-effects such as bone pain [13,14],

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fever, myalgia, and headache being associated with the treatment. However, animal and *in vitro* studies have described more serious side-effects associated with rhG-CSF treatment such as leucocytosis [15], and stimulation of cultured leukemia cells [16,17]. From this latter evidence, it appears essential that data should be collected on the distribution of this recombinant cytokine in human body fluids in order to monitor its bioactivity. However, to date few studies have reported on the pharmacokinetics of this drug and its distribution in the human body [18,19].

In the present study, a high-performance immunoaffinity chromatographic (HPIAC) technique was used to measure rhG-CSF concentrations in three different human body fluids at maximum plasma half-life in patients with acute lymphoblastic leukemia, undergoing rhG-CSF treatment for chemotherapy-induced neutropenia.

2. Experimental

2.1. Materials

Solid glass beads (5–10 μm diameter) and 3-aminopropyl-triethoxysilane were obtained from Polysciences (Warrington, PA, USA). 1,1'-Carbonyldiimidazole and all other laboratory chemicals were obtained from Sigma Chemical Company (St. Louis, MO, USA). Streptavidin was purchased as analytical grade, lyophilized product from Bethesda Research Laboratories (Gaithersburg, MD, USA) and reconstituted in 50 m*M* carbonate buffer (pH 9.0). Recombinant human granulocyte colony stimulating factor (rhG-CSF) was obtained from Amgen (Thousand Oaks, CA, USA). Recombinant human granulocyte-macrophage-colony stimulating factor (rhGM-CSF), macrophage-colony stimulating factor (rhM-CSF), and erythropoietin (rhEPO) were obtained from Genzyme Corporation (Bedford, MA, USA).

2.2. Patient and control samples

The patient group was composed of 8 males and 4 females, aged 32–45 years, with clinically proven acute lymphoblastic leukemia (ALL), who were enrolled in a rhG-CSF protocol following severe chemotherapy-induced neutropenia (200 μg/m^2 rhG-CSF daily). All of the patients studied were on day 10 of their treatment protocol. Control groups of 10 ALL patients (age and sex matched) receiving only chemotherapy, due to minimal neutropenic changes, and 10 healthy individuals were also included in the study. Informed consent was obtained from all subjects in the three different study groups. Bone marrow aspirate fluid (BMAF), and cerebral-spinal fluid (SF) samples were taken from all ALL patients at 5 h post-treatment (peak plasma concentration [13]) for routine analysis and immunoaffinity measurements. In addition, plasma samples were obtained hourly from the cytokine-treated patients and at a time equal to 5 h post-treatment from all of the subjects. The samples were collected in sterile glass vacutainer tubes (containing buffered sodium citrate; Becton-Dickinson, Rutherford, NJ, USA) and clarified by centrifugation at 1500 *g* for 2 min in a Beckman microfuge.

2.3. Preparation of murine anti-rhG-CSF antibody

Murine monoclonal antibody (MAb) reactive with rhG-CSF was produced by the technique of Köhler *et al.* [20]. Prior to immobilization in the HPIAC column, the MAbs were isolated from ascites fluid by ion-exchange chromatography and absorbed against a panel of growth factors, including naturally-occurring G-CSF, rhGM-CSF, rhM-CSF, and rhEPO, to remove possible cross-reactivity. The specificity of the purified MAb was tested by Western blot analysis [21].

2.4. Preparation of streptavidin-coated beads

MAb-coated glass beads were prepared by modifying the surface of 1 g of acid-washed beads by refluxing them in a 10% (v/v) solution of 3-amino-propyltriethoxysilane dissolved in toluene for 16 h followed by attachment of carbonyldiimidazole groups to the silanized bead

surface, as previously described [22]. The beads were recovered, thoroughly washed in dioxane, air-dried and immediately coated with streptavidin by suspending 1 g of beads in 10 ml of 50 m*M* carbonate buffer pH 9.0, containing 1 mg of streptavidin. This mixture was incubated for 18 h at 4°C on an oscillating shaker, before recovering the beads, washing them five times in 0.01 *M* phosphate buffer pH 7.0, by sedimentation. Finally the beads were stored in 0.01 *M* phosphate pH 7.0 at 4°C.

2.5. Preparation of immunoaffinity columns

Anti-rhG-CSF MAbs were modified by attaching biotinamido-caproylhydrazide to metaperiodate oxidized carbohydrate moieties in their Fc portions as previously described [22–25]. The biotinylated MAbs were immobilized onto the surface of the streptavidin-coated beads by incubating 500 μl of antibody (200 μg dissolved in 0.01 *M* phosphate buffer pH 7.0) with 1 g of beads overnight at 4°C. The beads were then washed five times in 0.1 *M* phosphate buffer pH 7.0, slurry-packed into 50 × 4.6 mm I.D. polyether-ether-ketone (PEEK) biocompatible chromatography columns (Alltech Associates, Deerfield, IL, USA) and attached to the HPLC system.

2.6. Instrumentation

HPIAC separations were performed on a Beckman 340 isocratic HPLC system (Beckman Instruments, San Ramon, CA, USA), equipped with a Beckman Model 112 pump, a Model FL-1 fluorescence detector (Rainin Instrument Company, Emeryville, CA, USA–λ_{ex} = 340 nm; λ_{em} = 450 nm; attenuation 32) and a Shimadzu C-R3B recording peak integrator (Shimadzu Scientific Instruments, Columbia, MD, USA). The elution profile was automatically controlled by a Model III OPG/S solvent selector/gradient controller (Autochrom, Milford, MA, USA). Samples were introduced into the system by injection through an Altex 210 injection port, equipped with a 100-μl sample loop.

2.7. Preparation of rhG-CSF standards

Standards of 250, 500, 1000, and 2000 pg/ml rhG-CSF were prepared by dissolving a stock solution of rhG-CSF (containing 1 μg/ml) in 0.01 *M* phosphate buffer pH 7.0. Known volumes of the stock solution were also added to normal plasma, BMAF, and SF to make final solutions which contained 100, 200, 400, 800, and 1600 pg/ml of rhG-CSF.

2.8. Immunoaffinity isolation of rhG-CSF

All samples were derivatized with *o*-phthalaldehyde prior to immunoaffinity analysis by the technique of Schlabach and Wehr [26]. Following injection of the sample, the column was isocratically developed with 0.01 *M* phosphate buffer pH 7.0 for 5 min at a flow-rate of 1.0 ml/min. Following the initial 5 min run, during which the rhG-CSF bound to the immobilized antibody, elution was started. A pH gradient (pH 7.0–1.5) was developed by adding 0.1 *M* glycine-HCl [19,20] to the running buffer over a further 5 min and the upper limit of the gradient was maintained for a further 2 min, before recycling the column by returning it to the initial running conditions. Throughout the entire run, the column temperature was maintained at 4°C by a glass column jacket, attached to a recycling ice-bath. Fractions of the eluted material (50 μl) were collected in 400-μl Beckman microfuge tubes, in a modified ISCO Cygnet fraction collector (ISCO, Lincoln, NB, USA) equipped with a plastic turntable capable of holding the Beckman tubes. Each fraction was carefully pipetted into a dialysis chamber in a 28-well GibcoBRL microdialysis system, equipped with a 6000–8000 Da cut-off membrane (Life Technologies, Gaithersburg, MD, USA) and dialyzed for 30 min against a continuous flow of 0.01 *M* phosphate pH 7.0. These fractions were used in the enzyme immunoassay, described below, to check the validity of the HPIAC technique.

2.9. Enzyme immunoassay (EIA) for human rhG-CSF

rhG-CSF concentrations, were detected by the

technique of Sallorfors and Olofsson [27] using the anti-rhG-CSF MAb as the capture antibody and a commercially available rabbit anti-rhG-CSF antibody (R and D Systems, Minneapolis, MN, USA) and alkaline phosphatase-labelled goat anti-rabbit antibody (Sigma) as the detection system.

3. Results and discussion

Immunochemical analysis of the anti-rhG-CSF MAb revealed that it reacted against rhG-CSF, not against either the natural or other recombinant haematological growth factors or cytokines tested. HPIAC columns used, in the present study, to perform rhG-CSF measurements contained between 27 and 35 μg of immobilized MAb per column and when kept at a constant 4°C, the columns were able to perform approximately 200 analyses before the immobilized MAb lost its bioactivity.

HPIAC isolations produce the typical twin peak elution profile shown in Fig. 1. The first peak is composed of material unreactive with the immobilized antibody, while the second (peak B) contains the immunoaffinity isolated analyte. The chromatogram shown in Fig. 1 represents an HPIAC analysis of a BMAF sample taken from a rhG-CSF-treated patient. Similar chromatograms were obtained when other body fluids were analyzed by the HPIAC column, although the height of the second peak fluctuated with the amount of analyte recovered. Fig. 2 demonstrates the ability of the HPIAC system to detect different rhG-CSF standards, in non-proteinaceous medium and Table 1 shows the precision and recovery efficiency of the technique. Analysis of the data presented in Table 1 indicated that the technique was able to reproducibly detect rhG-CSF concentrations down to the 100 pg/ml level in all of the body fluids tested. Analysis of "spiked" samples revealed that the most reproducible levels were observed in the BMAF with recovery levels of 90.0% at the 100 pg level to 99.3% at the 1600 pg level and coefficients of variation (C.V.) ranging from 13.33 to 2.2. Spinal fluid samples also gave reasonable results with recoveries ranging from 95.0% (C.V. 6.32) at the 100 pg level to 97.5% (C.V. 2.88) at the 1600 pg level. Plasma concentrations were also reliable, with recovery, accuracy and C.V. similar to those demonstrated for SF samples.

Plasma studies (not shown) confirmed the earlier report of Yoshida *et al.* [13] that the maximum peak plasma concentration of rhG-CSF in leukemia patients receiving 200 $\mu g/m^2$ of

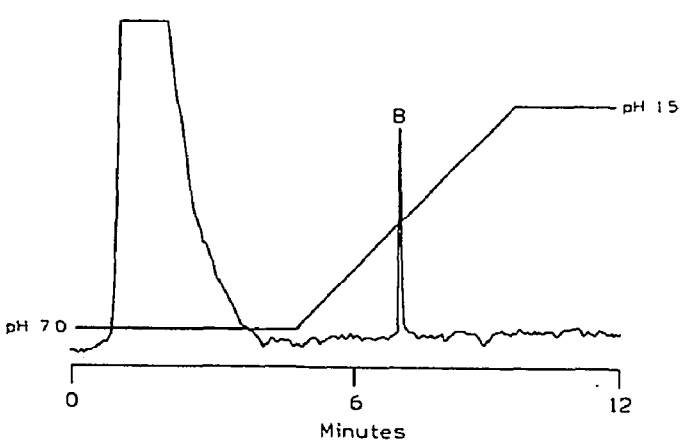

Fig. 1. HPIAC isolation (peak B) of rhG-CSF from a 5-h post-treatment rhG-CSF-treated ALL patient's bone marrow aspirate fluid. Flow-rate: 0.5 ml/min; fluorescence detection at $\lambda_{em} = 450$ nm, attenuation 32; running buffer: 0.01 *M* phosphate buffer pH 7.0. The pH elution gradient is indicated by the additional line on the chromatogram.

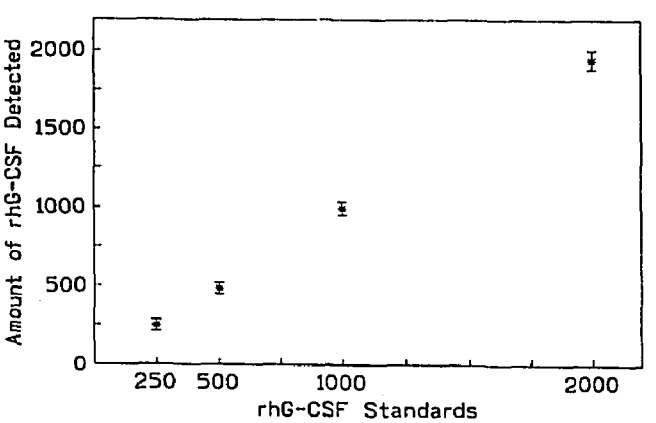

Fig. 2. Calibration curve produced by passing a series of rhG-CSF standards, in non-proteinaceous buffer, through the HPIAC column under the same running conditions as described in Fig. 1. Each point represents the mean ± S.D. of 5 repeat determinations.

Table 1
Precision and recovery of rhG-CSF from different body fluids ($n = 10$)

Amount added (pg/ml)	Amount recovered ±S.D. (pg/ml)	C.V. (%)	Recovery (%)
Plasma			
100	92 ± 8	8.7	92.0
200	183 ± 17	9.29	91.5
400	388 ± 25	6.44	97.0
800	769 ± 28	3.64	96.1
1600	1555 ± 36	2.32	97.2
BMAF			
100	90 ± 12	13.33	90.0
200	186 ± 10	5.38	93.0
400	391 ± 18	4.60	97.8
800	790 ± 26	3.29	98.8
1600	1589 ± 35	2.20	99.3
SF			
100	95 ± 6	6.32	95.0
200	193 ± 15	7.77	96.5
400	384 ± 29	7.55	96.0
800	775 ± 32	4.13	96.9
1600	1560 ± 45	2.88	97.5

Table 2
Distribution of rhG-CSF in body fluids of the study groups at peak plasma concentration (5 h post-treatment)

Study group	rhG-CSF range (pg/ml)
rhG-CSF-Treated ALL	
Plasma	1503 ± 229
BMAF	2660 ± 252
SF	838 ± 83
Non-treated ALL	
Plasma	< 100
BMAF	< 100
SF	< 100
Normal controls	
Plasma	< 100

rhG-CSF was 4.5–5.0 h post-treatment. This time frame is also compatible with the studies performed in children by Stute *et al.* [18] and Kearns *et al.* [19]. However, all of these other studies used plasma as the test material and did not report rhG-CSF levels in other body fluids.

The distribution of rhG-CSF in the different body fluids of rhG-CSF-treated ALL patients is given in Table 2. At 5 h post-treatment, the highest concentration of the drug was found in the bone marrow aspirate fluid, with levels reaching 2660 ± 252 pg/ml. Plasma concentrations only reached half of those recorded in the BMAF; SF concentrations were approximately one third of those found in the BMAF. However, the SF concentrations were still high and could contribute to the fever and headaches associated with this treatment regime [28]. Detectable levels of rhG-CSF could not be found in any body fluids obtained from non-rhG-CSF-treated ALL patients or in normal controls. This finding is in keeping with the data of Sallerfors and Olofsson [27], who reported plasma levels of less than 0.1 ng/ml in all of their non-treated ALL patients and normal controls.

Comparison between the concentrations of rhG-CSF, detected by HPIAC, and a conventional EIA was made in order to evaluate the efficacy of the immunoaffinity technique. Analysis of the results obtained from the two assays demonstrated a close correlation when using SF samples but not when comparing BMAF or plasma samples (Fig. 3). Linear regression analysis of the SF levels detected by both techniques demonstrated a regression line with a slope of 1.0275 ± 0.045 and an r value of 0.9905. However, such a close correlation could not be achieved when testing the other two body fluids. Further analysis demonstrated that this lack of correlation between the two techniques arose from interference of endogenous growth factor present in these fluids. In our hands, the EIA was unable to distinguish between recombinant and natural cytokine thus accounting for the differences seen when testing these body fluids.

The studies described above show that HPIAC is a versatile technique which can be applied to the isolation and analysis of any biological material, provided a specific antibody is either readily available from a commercial source or can be manufactured in the laboratory. The application of such antibodies allowed the detection of recombinant growth factor without hin-

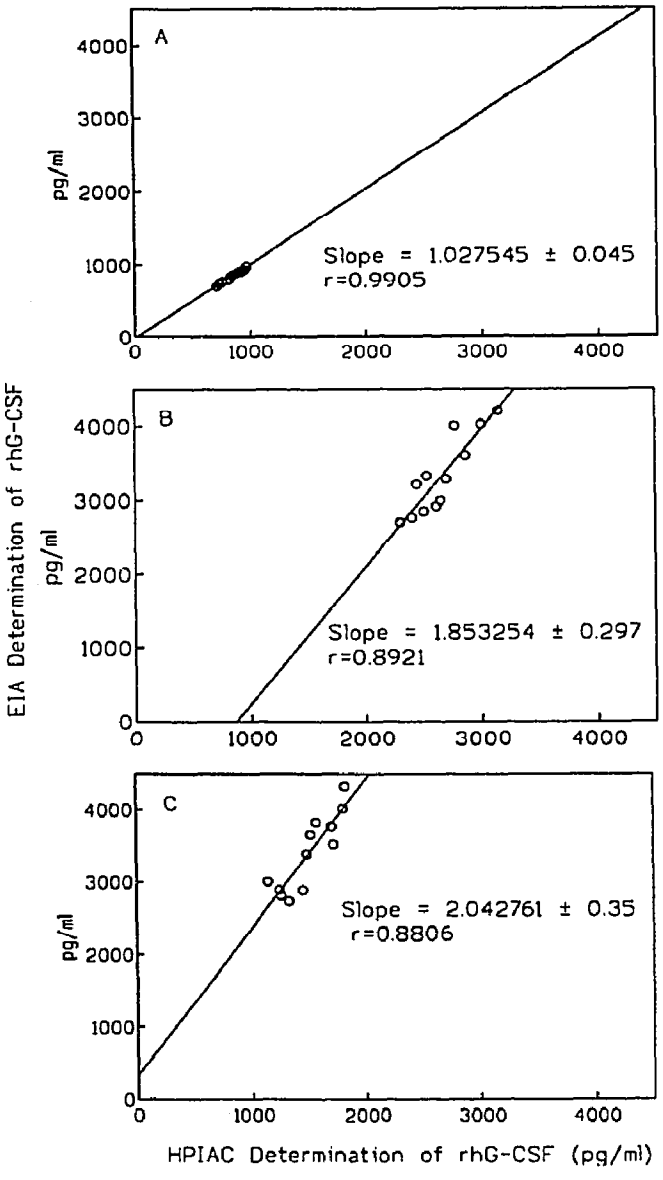

Fig. 3. Concentrations of rhG-CSF in different body fluids obtained from the 12 cytokine-treated patients detected by HPIAC and EIA at peak plasma concentration. (A) Cerebral-spinal fluid samples, (B) bone marrow aspirate fluid samples, and (C) plasma samples. The line in each graph represents the least squares linear regression analysis of the data. The y-intercept for graph A = $-20.83758x \pm 37.92416$, graph B = $-1601.346x \pm 792.9088$, and graph C = $333.0487x \pm 528.1232$.

derance from naturally occurring materials; an often serious technical problem associated with many immunoassays. Although this problem was encountered in this study when comparing immunoaffinity to a conventional immunoassay, generally HPIAC compares well with other immunological assays [24,29] and can be applied to the detection of analytes in human biological fluids.

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