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Short communication

High-performance liquid chromatographic purification and capillary electrophoresis quantification of the chemokine stromal cell-derived factor-1

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Abstract

Chemokines are members of the chemotactic cytokines family implicated in various immunoregulatory functions. The CXC–chemokine stromal cell derived factor-1 (SDF-1α) was purified from the culture medium of murine bone marrow stromal cell line (MS-5) by affinity and reversed-phase liquid chromatography. Yield and purity were assessed by capillary electrophoresis (CE) with reference to the human SDF-1α from recombinant DNA technology. CE technique was useful to evaluate the purity of human SDF-1α from chemical synthesis and to resolve murine and human SDF-1α, differing by only one amino acid. Chemotactic activity of the murine SDF-1α was tested on the basis of CE quantification.

Keywords: Chemokines; SDF-1α

1. Introduction

Chemokines are a large family of small (about 70 amino acids), structurally similar cytokines, implicated in a variety of immunoregulatory functions and pathological conditions [1,2], activating leukocytes and directing them along a concentration gradient into lymphoid and non-lymphoid tissues. Stromal cell-derived factor-1 (SDF-1α) is a member of the CXC-group of chemokines and is constitutively expressed in bone marrow stromal cells [3,4]. SDF-1α acts as a chemoattractant on lymphocytes, monocytes [5] and hematopoietic progenitor cells [6] and has been recently shown to block HIV-1 entry mediated by the receptor CXCR-4 [7,8].

The first aim of this study was to obtain highly purified SDF-1α from a murine bone marrow stromal cell line (MS-5) [9] to complete the studies on its role in normal and pathological hematopoiesis [6]. Here we report the multi-step purification strategy used for this purpose, modified with respect to a previously published one [5]. Both affinity and reversed-phase liquid chromatography (HPLC) were carried out using the same HPLC apparatus, conveniently modified. Moreover, ion-exchange chromatography, used by others as an intermediate step [5], was replaced by ultrafiltration.

The second objective was to assess the yield and

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purity of the purified murine SDF-1α by capillary electrophoresis (CE) with reference to the standard human SDF-1α by recombinant DNA technology. After quantification, murine SDF-1α was tested in chemotactic assays and its activity resulted comparable to that of the human recombinant and the human chemically synthesised proteins. CE allowed to identify the presence of impurities in the standard SDF-1α from different sources and, under improved analytical conditions, to achieve the baseline resolution of human and murine SDF-1α, differing by only one amino acid.

2. Experimental

2.1. Standard proteins

Chemically synthesised human SDF-1α (1–67) was kindly donated by I. Clark-Lewis (Biomedical Research Centre, University of British Columbia, Vancouver, Canada) [7,10]. The lyophilised product was resuspended in water to a final concentration of 1 mg/ml (according to the weight reported on the tube) and aliquots stored at −80°C. Working standard solutions were obtained by diluting the stock solution with water.

Human recombinant SDF-1α expressed in *E. coli* by recombinant DNA technology was purchased from PeproTech EC (London, UK). The lyophilised powder was resuspended in water to a final concentration of 100 μg/ml according to the purity claimed by the manufacturer (>98%).

2.2. Cell culture

A murine bone marrow stromal cell line (MS-5) [9] was cultured in roller bottles in 150 ml of Iscove's modified Dulbecco's medium (containing 10% FCS) until it reached confluence. The medium was then replaced with 250 ml of X-VIVO serum-free medium (BioWhittaker, Walkersville, MD, USA) and supernatants collected every 5 days.

2.3. HPLC purification of murine SDF-1α

Murine SDF-1α was purified modifying a previously described scheme [5]. The MS-5 conditioned medium (1100–2000 ml) was filtered through 0.22 μm membrane and applied to a HiTrap Heparin affinity column (5 ml, loading capacity 3 mg Antithrombin III/ml gel, according to the manufacturer) (Pharmacia, Uppsala, Sweden) employing an HPLC system (Beckman Instruments, Palo Alto, CA, USA) with two dual piston pump, UV absorbance detector and computer controlled by System Gold software. The column, stored in ethanol 20%, was washed with 20 ml of 10 m*M* sodium phosphate (pH 7.3) before sample loading via the HPLC pump. To avoid a possible backpressure enhancement due to the high protein concentration of the medium, the column was directly connected to the pump outlet check-valve by-passing the mixer and the other on-line filters. The HiTrap column and the mobile phase reservoirs were kept on ice to prevent possible protein denaturation and the SDF-1α loaded onto the column pumping the cell culture supernatant at 2.0 ml/min and allowing to recirculate twice. The column was washed with 25 ml of 10 m*M* sodium phosphate (pH 7.3), and then SDF-1α eluted by NaCl gradient (0.4–2.0 *M*) in phosphate buffer, at a flow-rate of 0.3 ml/min monitoring at 280 nm and collecting fractions (3 ml) every 10 min. The fractions displaying chemotactic activity on human peripheral lymphocytes, were pooled (12 ml), desalted and concentrated by ultrafiltration (5 h, 4000 *g*) in Centricon-3 (Amicon, Beverly, MA, USA). The retentate (1.5 ml) was further concentrated (600 μl) under vacuum (Savant Instruments, Hicksville, NY, USA) and injected 100 μl at a time onto a C_4 RP-analytical column (250×4.6 mm I. D., 5 μm particle size, 300 Å) (Vydac, Hesperia, CA, USA). SDF-1α was eluted with a gradient of acetonitrile in 0.1% TFA (0–100% in 60 min) at 1.0 ml/min, monitoring at 280 nm and collecting 1 ml fractions during the analysis. The fraction displaying chemotactic activity, were pooled, lyophilised, resuspended in 10 m*M* phosphate buffer (150 m*M* NaCl) (400 μl) and used for CE analysis and biological assays.

2.4. Capillary electrophoresis

A P/ACE 5010 apparatus (Beckman) was used, with an uncoated fused-silica capillary of 27 cm total length (20 cm to the detector×50 μm I.D., 375 μm O.D.), and 50 m*M* sodium phosphate (pH 5.0) – 50

m*M* SDS (1:1, v/v) as running buffer. The applied voltage was +15 kV (about 67 μA), with the UV absorbance detector set to 200 nm and the temperature at 20°C. A coated capillary (CElect P150, Supelco, Bellefonte, PA, USA) of 27 cm total length (20 cm to the detector×50 μm I.D., 363 μm O.D.), was also tested with *N*-[Tris(hydroxymethyl)methyl]-3 aminopropanesulfonic acid (TAPS) 100 m*M*, pH 11.5 as running buffer, and the potential at +9 kV (55 μA). The capillaries were assembled in the P/ACE cartridge format (100×200 μm aperture) and samples injected by pressure (3.45 kPa) for 10 s (about 22 nl). Between injections both the capillaries were rinsed under high pressure with 0.1 *M* NaOH (3 min) followed by the running buffer (3 min). The peak area corrected for the migration time (t_M) was always considered for quantitative analysis.

2.5. Proteins quantification

The proteins recovered after each purification step starting from stromal cells culture medium were determined by BioRad Protein Assay (BioRad Laboratories, Munich Germany). The final purified product (murine SDF-1α) was quantified by CE assuming as a reference the response factor of the standard human SDF-1α from recombinant DNA. The standard solution was injected daily and the mean (n=5) area/pmol ratio used to quantify the unknown purified protein.

2.6. Chemotaxis assay

Assays were performed with human peripheral blood lymphocytes as previously described [5]. The activity of SDF-1α was expressed as percentage of migrating cells.

3. Results

The fractions purified from MS-5 by heparin column, which resulted active by the chemotactic assay, were ultrafiltered, concentrated and analysed by HPLC. Chemoactractant activity was found associated to a relevant peak with retention time (t_R) of 24.47±0.04 min (CV%=0.16%, n=5) which was collected and dried before quantification and characterisation by CE.

In Fig. 1 are reported the electropherograms obtained analysing in an uncoated capillary the human SDF-1α by recombinant DNA technology, by chemical synthesis and the murine SDF-1α purified by us. Migration times (t_M) were 4.43±0.22 min, 4.89±0.14 min and 5.15±0.21 min (n=5), respectively. The human SDF-1α obtained by chemical synthesis revealed the presence of the expected polypeptide together with various synthesis by-products not completely resolved from the major peak and probably related to incomplete amino acid coupling reactions [11]. The recombinant human SDF-1α showed an additional peak with t_M 6.13 min, corresponding to 22% of the total area and displaying similar absorbance characteristics of the main peak. This was taken into account to generate a calibration factor able to quantify the murine purified SDF-1α (2.05±0.033 corrected peak area/ng, n=4). From 2.0 l of crude murine stromal cells medium, 28 μg of SDF-1α were obtained corresponding to a specific activity of 6071 U/mg, with a 1:2428-fold purification rate (Table 1). The CE profile of this product (Fig. 1) showed only one relevant peak, so confirming that the purity requirements have been fulfilled.

The biological activity assay on the human synthetic, human recombinant and murine SDF-1α was set up on the basis of the concentrations evaluated by CE, thus avoiding time-consuming experiments to find out the best conditions for the maximum cell migration. The assay showed a linear and proportional decrease of cell migration relative to the protein dilution independent of the source of SDF-1α (Fig. 2).

As human and murine SDF-1α differ only for 1 amino acid (Val^{18} in humans is replaced by Ile^{18} in mice) CE analysis needed to be optimised to reach the resolution of the two proteins. Fig. 3 shows that the baseline separation was achieved by using an aqueous pH 11.5 buffer and a neutral coated capillary with minimised interactions between the proteins and the capillary wall [12]. Individual injections of human recombinant and murine SDF-1α gave t_M of 4.89±0.03 min and 5.45 ±0.04 min (n=3), respectively. Under these conditions, the human synthetic SDF-1α had a t_M of 4.48±0.03 min

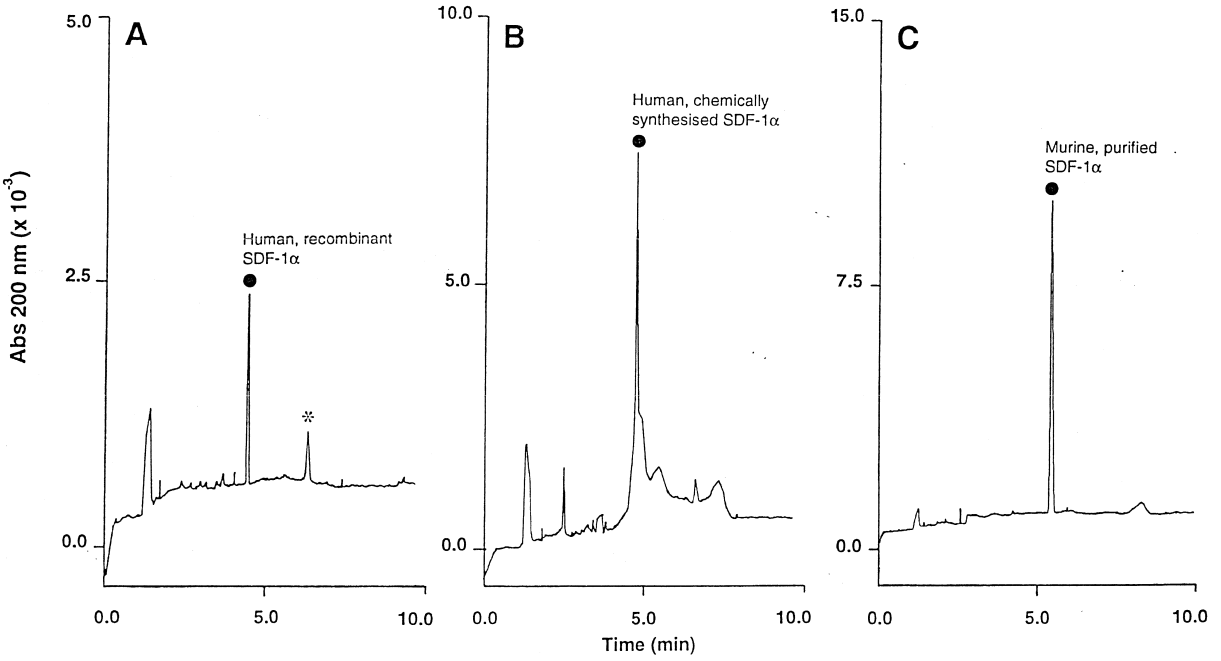

Fig. 1. Electropherograms of SDF-1α from different sources, analysed on uncoated capillary (20 cm to the detector×50 μm I.D.). Running buffer: 50 m*M* sodium phosphate (Na_2HPO_4) (pH 5.0): 50 mM SDS (1:1, v/v). Injection by pressure mode for 10 s. (A) Human, from recombinant DNA technology. (B) Human, from chemical synthesis. (C) Murine, purified from MS-5 cell supernatant. *=Interfering peak.

and a large electroosmotic flow (EOF) peak (data not shown) probably comprising the unknown synthesis by-products seen in Fig. 1. The calibration factor derived from the synthetic SDF-1α standard gave a murine SDF-1α final yield of 30.5±0.17 μg, not significantly different (Student's "*t*" test) from the value in Table 1 based on quantification with SDF-1α from recombinant DNA technology.

4. Discussion

The biochemical approach here described for SDF-1α purification from MS-5 cell line supernatant employs only one HPLC apparatus for both affinity and reversed-phase chromatography, and an ultrafiltration step for simultaneous concentration and desalting of the sample. This results in a cost effective

Table 1
Purification of murine stromal derived factor (SDF-1α)

	Volume (ml)	Chemotactic activity (U)	Protein amount (mg)[a]	Specific activity (U/mg)	Purification (fold)	Yield (%)
MS-5 supernatant	2000	10.000	4060	2.5	1	100
Heparin HiTrap	12.0	1920	8.16	253.3	96	19
Ultrafiltration	0.6	360	0.63	571.4	232	3.6
RP-HPLC	0.4	170	0.028[b]	6071	2428	1.7

[a] Proteins were assayed by an unspecific colorimetric method (BioRad Protein assay).
[b] Specific quantification was carried out by CE analysis based on human recombinant SDF-1α as standard protein, taking into account its purity.

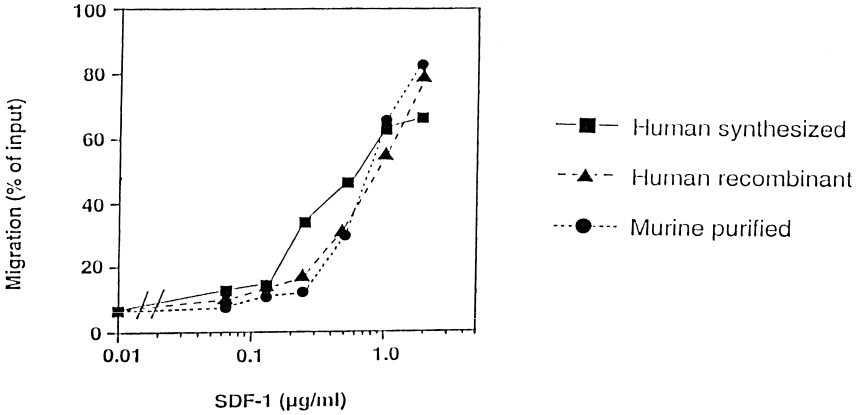

Fig. 2. Chemotactic activity assay on SDF-1α from different sources. Human peripheral blood lymphocytes (3×10^5) were loaded into chemotactic chambers containing serial dilutions of SDF-1α from either human synthesised, human recombinant and purified murine SDF-1α. The percentage of cell migrating was measured by FACS after 3 h.

and time-saving procedure, with a final product characterised by high purity and specific activity. The amount of the protein recovered at the end of the process was quantified by CE, a more specific method than the commonly employed colorimetric assays, thus appearing slightly lower than the one obtained by Bleul et al. [5].

The effectiveness of CE for resolution of proteins

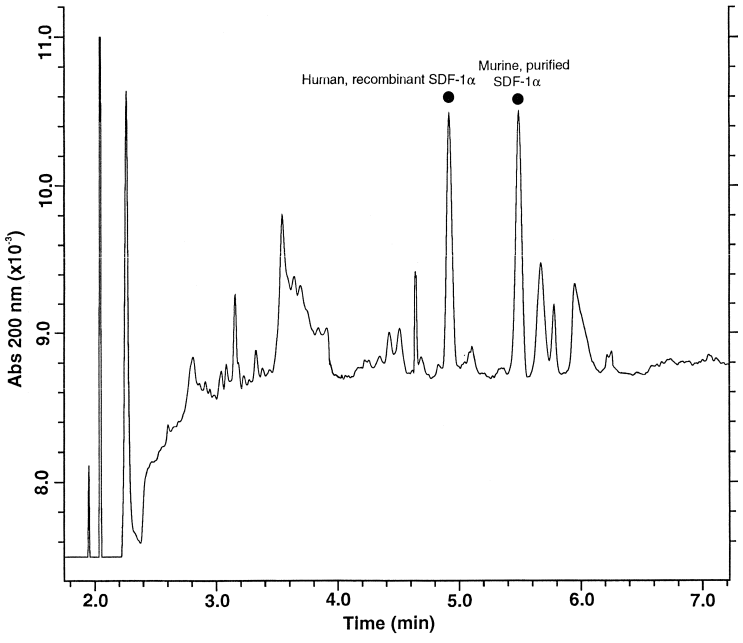

Fig. 3. Capillary electrophoresis separation of the human recombinant and murine stromal purified SDF-1α. The mixture of the two proteins was injected for 10 s in the P150 CElect coated capillary with TAPS 100 m*M*, pH 11.5 as running buffer and 9 kV applied voltage.

having different contents of hydrophobic residues employing a mixture aqueous–organic buffer with suitable amounts of zwitterionic agents was already proved [13]. In our work human and murine SDF-1α, differing only by a single amino acid replacement resulted separated by more than 1 min in a neutral hydrophilic coated capillary with TAPS–diethylentriamine–acetonitrile (45:45:10, v/v/v) as running buffer (data not shown). However, baseline resolution of the two proteins differing only by 14 Da, was also achieved simply employing aqueous TAPS, pH 11.5 as running buffer and reducing the applied potential from 12 to 9 kV (Fig. 3).

To our knowledge, analytical conditions to separate murine and human SDF-1α have never been described before, and may be useful to study the production of SDF-1α by different cell types, as well as to discriminate between alternatively spliced forms of SDF-1 (α and β) differing in the carboxyl-terminal four amino acid residues [3,5]. Moreover, in view of developing mouse models to study SDF-1 based gene therapy approaches to HIV-1, CE may be important in discriminating between SDF-1 produced by endogenous (murine) and genetically modified cells (human). CE analysis allowed also the identification of degradation/interfering products in the standard proteins by different sources, thus confirming its utility when coupled to HPLC and SDS-PAGE in the Quality Control of proteins production.

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