# Multimerization of monocyte chemoattractant protein-1 is not required for glycosaminoglycan-dependent transendothelial chemotaxis

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Chemokines interact with specific G-protein-coupled cell-surface receptors and with glycosaminoglycans (GAGs), such as heparan sulphate. Although chemokines often form multimers in solution, this process may be enhanced following interaction with GAGs on the cell surface, or within the extracellular matrix. However, the significance of multimerization for chemokine function remains controversial. In the present study, a fusion protein was prepared between the prototypical human CC chemokine, monocyte chemoattractant protein-1 (MCP-1; also known as CCL-2) and a large secreted placental alkaline phosphatase (SEAP) moiety. This fusion protein (MCP-1–SEAP) remained monomeric under conditions that promote oligomerization of the native chemokine. Radioligand binding showed that both native MCP-1 and MCP-1–SEAP competed for the same site on the surface of HEK-293 cells expressing the CCR2b chemokine receptor. The interaction between either chemokine species and endothelial cell surface GAGs was antagonized by the addition of the heparan sulphate-like molecule, heparin. Both MCP-1 and MCP-1–SEAP induced a $Ca^{2+}$-flux in the THP-1 monocytic cell line, and were equally effective at promoting transendothelial chemotaxis of mononuclear immune cells, with maximal migration being produced by treatment with 12 nM of either species. In each case this chemotactic response was almost completely antagonized by the addition of heparin. The importance of interaction between either native MCP-1 or MCP-1–SEAP and cell-surface GAGs for transcellular migration was demonstrated by the almost complete absence of leucocyte chemotaxis across monolayers of GAG-deficient mutant cells. In summary, this study shows that multimerization is neither necessary for, nor potentiates, the biological activity of MCP-1. However, the results do clearly demonstrate the importance of the interaction between MCP-1 and cell-surface heparan sulphate for transmonolayer leucocyte chemotaxis.

Key words: heparan sulphate, heparin, MCP-1, secreted placental alkaline phosphatase fusion protein.

## INTRODUCTION

Monocyte chemoattractant protein-1 (MCP-1; also known as CCL-2) is a member of the chemokine family of cytokines, which have a range of functions including the recruitment of specific leucocyte subsets to sites of inflammation [1]. MCP-1 is produced by a variety of cell types, including lymphocytes, mononuclear phagocytes and vascular endothelial cells. It is expressed in response to diverse stimuli, including the cytokines platelet-derived growth factor, interferon-$\gamma$ (IFN-$\gamma$), interleukin-1$\beta$, tumour necrosis factor-$\alpha$ (TNF-$\alpha$), granulocyte/macrophage colony-stimulating factor and macrophage colony-stimulating factor, as well as hypoxia and superoxides. Inappropriate MCP-1 expression is associated with a number of pathological inflammatory states, including rheumatoid arthritis [2,3], asthma [4] and the acute rejection of allografted organs [5].

The *MCP*-1 gene promoter contains binding sites for the transcription factors activator protein-1 ('AP-1'), octamer transcription factor and nuclear factor $\kappa$B ('NF$\kappa$B'; see [6]), which is thought to be responsible for induction of expression. MCP-1 mediates the recruitment of T lymphocytes and monocytes to inflammatory sites [7]. This attraction is mediated through the G-protein-coupled receptor CCR2, which is found as two alternatively spliced isoforms, CCR2a and CCR2b [8]. Site-directed and truncation mutagenesis have allowed the identification of the sequence domains, including $Tyr^{13}$, on MCP-1 that are important for the activation of CCR2 [9]. In addition, it is also known that MCP-1 can induce CCR2 dimerization [10]. It has been shown that phosphorylation of serine and threonine residues in the C-terminal cytoplasmic tail of CCR2b, and resultant receptor internalization, are not involved in chemotaxis, but a role for G-protein signalling has been suggested [11], which appears to involve the MAPK (mitogen-activated protein kinase) pathway [12].

Mature MCP-1 is secreted as a 76-amino-acid protein with two internal disulphide bridges between conserved cysteine residues ($Cys^{34}$–$Cys^{59}$ and $Cys^{35}$–$Cys^{79}$). The adjacent nature of the $Cys^{34}$ and $Cys^{35}$ residues involved in this interaction defines MCP-1 as a member of the CC or $\beta$ chemokine subclass, whose members include macrophage inflammatory protein (MIP)-1$\alpha$, MIP-1$\beta$ and RANTES (regulated upon activation normal T-cell expressed and secreted). NMR studies of MCP-1 [13], and the homologous CC chemokine MIP-1$\beta$ [14], suggest that MCP-1 has a prominent mobile N-terminus and a stable core region of three anti-parallel $\beta$-sheets overlaid with a C-terminal $\alpha$-helix. Additional studies on the basis of sedimentation equilibrium ultracentrifugation and chemical cross-linking [15] have shown that, at high concentrations, MCP-1 can dimerize through an interaction of N-terminal amino acids [16,17]. This observation is supported by X-ray crystallographic and NMR solutions for the H-bond-stabilized dimers [13,18].

During acute inflammation, local chemokine concentrations can reach concentrations of several ng/ml [19,20]. However, even at this concentration, free chemokines are likely to exist predominantly in a monomeric form [21]; indeed, the dissociation constant for MCP-1 aggregates has been estimated to be within the range of 0.5–33 $\mu$M [17,21]. A recent study of mutant variants of MCP-1 has confirmed the biological activity of the monomeric

Abbreviations used: CS-C, chondroitin sulphate-C; EMEM, Eagle's minimal essential medium; GAG, glycosaminoglycan; HBSS, Hanks balanced salt solution; IFN-$\gamma$, interferon-$\gamma$; IP-10, 10 kDa interferon-inducible protein; MCP-1, monocyte chemoattractant protein-1; MIP, macrophage inflammatory protein; PBMC, peripheral blood mononuclear cell; SEAP, secreted placental alkaline phosphatase; TNF-$\alpha$, tumour necrosis factor-$\alpha$.

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form in free solution [17]. However, the authors of this study suggest that MCP-1 multimerization may have a role in the sequestration of MCP-1 by glycosaminoglycan (GAG) components of the cell-surface, such as heparan sulphate. This heparin-like molecule constitutes up to 90 % of the total GAG on vascular endothelium [22]. The interactions among chemokines and GAGs may be essential for the formation of stable chemokine concentration gradients that promote vectorial leucocyte migration across the vascular endothelium at sites of inflammation.

To investigate the functional significance of chemokine multimerization, and to examine the role of higher-order species in interaction with the extracellular matrix, we produced a protein fusion between MCP-1 and the secreted placental alkaline phosphatase (SEAP; referred to as MCP-1–SEAP). This was constructed using a methodology similar to that described by Luster et al. [23], who 'fortuitously' produced a monomeric form of the normally aggregating chemokine, 10 kDa interferon-inducible protein (IP-10). The biological activity of our construct was compared with that of native MCP-1 using a range of biological assays designed to assess functional activity.

## MATERIALS AND METHODS

### Cell culture and characterization

The NIH 3T3 cell line (E.C.A.C.C. 88031146; E.C.A.C.C., Porton Down, Salisbury, Wilts, U.K.) and the monocytic cell line THP-1 (A.T.C.C. TIB-202; A.T.C.C., Manassas, VA, U.S.A.) were routinely grown in RPMI 1640 medium (Sigma, Poole, Dorset, U.K.). HMEC-1 cells (kindly provided by Dr Francisco Candal, Centers for Disease Control and Prevention, Atlanta, GA, U.S.A.) were propagated in MCDB-131 supplemented with 1 $\mu$g/ml cortisol and 10 ng/ml epidermal growth factor (Sigma). HEK-293 cells (E.C.A.C.C. 85120602; E.C.A.C.C.) were grown in Eagle's minimal essential medium (EMEM; Sigma) supplemented with non-essential amino acids. Wild-type CHO K1 and GAG-deficient 745-mutant CHO cells (kindly provided by Dr Jeffrey Esko, Department of Biochemistry and Molecular Genetics, University of Alabama at Birmingham, AL, U.S.A.) were cultured in Ham's F12 medium (Sigma). Media were all supplemented with 10 % (v/v) foetal calf serum (FCS), 50 $\mu$g/ml gentamicin and an antibiotic/antimycotic mixture (Life Technologies, Paisley, Renfrewshire, Scotland). Cells were maintained at 37 °C in a humidified atmosphere supplemented with 5 % $CO_2$.

The expression of heparan sulphate by both wild-type and 745-mutant CHO cells was assessed by immunofluorescence flow cytometry after primary labelling with an antibody specific for the N-sulphated glucosamine residue (Clone AB-10E4; Seikagaku, AMS Biotechnology Ltd., Abingdon, Oxon, U.K.). Analysis of the stained cells revealed that the wild-type cells expressed high levels of heparan sulphate ($P < 0.01$), but the fluorescence of stained 745-mutant cells was not significantly different from control levels ($P > 0.05$).

### Molecular cloning to create MCP-1–SEAP fusion

Standard molecular techniques were used throughout unless otherwise stated. MCP-1 expression was induced in 75 cm² flasks of confluent HMEC-1 cells by overnight treatment with 100 units/ml of both IFN-$\gamma$ and TNF-$\alpha$ (Peprotech, London, U.K.). Total RNA was prepared from these cells using RNAzol (Biogenesis, Bournemouth, Dorset, U.K.). cDNA was reverse-transcribed and used as a template for amplification of the MCP-1 coding region by PCR. Primers (5′: CGC AAG CTT CGG TCA AAC TGA AGC TCG CAC TCT CGC; 3′: CGC TCC GGA AGA TCT AGT CTT CGG AGT TTG GGT TTG C) were designed on the basis of the published MCP-1 gene sequence [24] and incorporated bases generating a 5′ *Hin*dIII restriction site. The 3′ primer differed from the genomic sequence such that the amplified MCP-1 product had the three bases of its stop codon removed and additionally encoded *Bgl*II and *Bsp*EI restriction sites in-frame with the chemokine open reading frame. After 30 cycles of low-stringency PCR, the products were electrophoresed and a 350 bp band was excised from a 2 % (w/v) agarose gel. Following purification on a Qiaquick column (Qiagen, Crawley, Sussex, U.K.), the modified MCP-1 cDNA was overhang-cloned into the pCR2.1 vector (Invitrogen, Groningen, The Netherlands). The fidelity of the amplified product was confirmed by sequencing. To create an in-frame MCP-1–SEAP fusion construct, the modified MCP-1 cDNA was subcloned into the pAP-Tag1 vector (kindly provided by Dr John Flanagan, Department of Cell Biology, Harvard Medical School, Harvard, NJ, U.S.A.) as a *Hin*dIII/*Bsp*EI fragment.

### Expression of MCP-1–SEAP

For efficient expression in mammalian cells, the MCP-1–SEAP open reading frame was subcloned into the vector pCDNA3.1Zeo+ between *Hin*dIII and *Xho*I (from pAP-Tag1) sites. NIH 3T3 cells were transfected with this expression construct using the Transfast reagent (Promega, Southampton, Hants, U.K.). Three days after transfection, 150 $\mu$g/ml zeocin (Invitrogen) was applied to select for stable transformants. After 3 weeks, individual zeocin-resistant NIH 3T3 colonies were isolated and expanded by the cloning cylinder technique. Clones were screened for MCP-1–SEAP expression by enzyme assay (see below) and the highest expressing clone, MCPSEAP.14, was chosen for further work.

For ligand binding and chemotaxis experiments, increased concentrations of MCP-1–SEAP were required than could be produced in the cell-culture supernatant. To achieve these concentrations, 75 cm² flasks of confluent MCPSEAP.14 cells were split into 150 cm² flasks and grown in 40 ml of serum-free Dulbecco's modified Eagle's medium (Life Technologies) supplemented with glutamine and antibiotics. After 3 weeks, this medium was recovered and centrifuged at 1000 ***g*** for 5 min to remove cellular debris. MCP-1–SEAP (approximate molecular mass, 78 kDa) was concentrated by ultrafiltration (Centricon Plus-80, 50 kDa limit; Millipore, Watford, U.K.).

### Assaying SEAP activity

Briefly, cell-culture medium was heated to 65 °C for 10 min to denature endogenous phosphatase activity, while preserving recombinant heat-stable SEAP activity. Extracts were centrifuged at 1200 ***g*** for 5 min and decanted supernatants were allowed to cool to room temperature. For enzyme assays, 100 $\mu$l of prepared cell-culture medium was mixed in a 96-well plate with an equal volume of *p*-nitrophenyl phosphate assay reagent. This was prepared using Fast pNPP (Sigma, Poole, Dorset, U.K.) at twice the manufacturer's recommended concentration, and, therefore, at the specified concentration in the final assay. The rate of $A_{410}$ change was monitored using a Dynatech 5000 microtitre plate reader (Dynex Laboratories, Ashford, Kent, U.K.).

### Calculating the specific enzyme activity of MCP-1–SEAP fusion protein

The concentration of MCP-1–SEAP in the heat-treated-cell-culture-medium supernatant from the MCPSEAP.14 clone was

measured by ELISA (R & D Systems, Abingdon, Oxon, U.K.). SEAP activity was measured colorimetrically in an aliquot of the same sample. To calculate the concentration of MCP-1–SEAP in samples prepared for ligand-binding and chemotaxis experiments, SEAP assays were performed and the chemokine fusion concentration was calculated from the specific enzyme activity of MCP-1–SEAP.

### Determination of molecular size

The potential for multimerization of native MCP-1 and of the MCP-1–SEAP fusion was assessed by SDS/PAGE electrophoresis and Western blotting, as described by Luster et al. [23]. Briefly, both samples were maintained at a concentration of 1 $\mu$M before mixing with sample buffer (0.125 M Tris, 2 % SDS, 10 % glycerol, 10 % 2-mercaptoethanol, 0.001 % Bromophenol Blue) and equal amounts of both proteins loaded on to 10 % (w/v) polyacrylamide gels. The proteins were then transferred on to Hybond-P membranes (Amersham Pharmacia Biotech, Amersham, Bucks, U.K.), which were blocked with Tris-buffered saline (10 mM Tris/HCl, pH 7.5, 150 mM NaCl; TBS) containing 5 % (w/v) BSA (Sigma) for 1 h. Membranes were incubated with anti-human MCP-1 antibody (1 $\mu$g/ml; R & D Systems) diluted in TBS for 12 h at 4 °C, washed in TBS containing 0.05 % v/v Tween-20, and a horseradish-peroxidase-labelled anti-goat IgG (0.4 $\mu$g/ml; Santa-Cruz, Santa Cruz, CA, U.S.A.) was added for 1 h at room temperature. After washing, the proteins were revealed using the enhanced chemiluminescence (ECL®) detection system (Amersham Pharmacia Biotech).

The potential of the MCP-1–SEAP fusion to form higher-order species was also assessed by gel filtration (FPLC; Amersham Pharmacia Biotech). To replicate physiological conditions, $^{125}$I-MCP-1 (specific radioactivity 81.4 TBq/mmol; New England Nuclear, Hounslow, Essex, U.K.) was diluted to a final concentration of 300 pM in Hanks balanced salt solution (HBSS), 10 mM Hepes, pH 7.4. The MCP-1–SEAP fusion protein was then added at a final concentration of 3 nM. The ability of GAGs to promote MCP-1 multimerization was assessed by addition to some aliquots of soluble heparin (Sigma) at a final concentration of 500 $\mu$g/ml. The samples were incubated at 37 °C for 2 h to allow any potential oligomers to form, and 200 $\mu$l aliquots were applied to Superdex 75 columns (Sigma) pre-equilibrated in HBSS, 10 mM Hepes, pH 7.4. The samples were resolved at a flow rate of 0.5 ml/min in the above buffer and the eluate was monitored by continuous flow spectrophotometry at 280 nm. Fractions (0.25 ml) were collected and counted for radioactivity on a Wallac Clinigamma counter (Wallac, Milton Keynes, U.K.). The columns were calibrated using the protein standards BSA (66 kDa), chymotrypsinogen A (25 kDa) and cytochrome *c* (12.5 kDa). The exclusion volume of the columns was determined using Dextran Blue (2000 kDa).

### Transfection of HEK-293 cells with CCR2b

HEK-293 cells were harvested from subconfluent culture by trypsinization, washed in serum-free EMEM and resuspended in the same medium at a concentration of approximately $1.5 \times 10^7$ cells/ml. Aliquots (400 $\mu$l) were placed in 0.4 cm electrode gap electroporation cuvettes (Bio-Rad, Hemel Hempstead, U.K.) and incubated on ice for 5 min with 10 $\mu$g of pCDNA3-FLAG-CCR2b (kindly provided by Professor Israel Charo, Gladstone Institute of Cardiovascular Disease, San Francisco, CA, U.S.A.). The sample was exposed to a potential difference of 500 V using Bio-Rad Gene Pulser II apparatus set to a capacitance of 50 $\mu$F. Cells were allowed to recover on ice for 10 min before being diluted in 20 ml of EMEM supplemented with non-essential amino acids and 10 % FCS.

Stable transfectants were selected by culture in medium supplemented with G418 (600 $\mu$g/ml; Calbiochem, Beeston, Notts, U.K.) and individual G418-resistant colonies were picked and expanded. Expression of CCR2b was assessed following labelling with a phycoerythrin-conjugated anti-CCR2b monoclonal antibody (R & D Systems) by immunofluorescence flow cytometry (FACScan; Becton Dickinson, Cowley, Oxon, U.K.).

### Ligand binding experiments

Binding of $^{125}$I-MCP-1 to HEK-CCR2b cells was assessed in 96-well plates. Cells were seeded in a volume of 300 $\mu$l and grown to confluence. Unlabelled ligand competition analysis was performed in a total volume of 150 $\mu$l by addition of $^{125}$I-MCP-1 diluted to 100 pM in binding buffer (HBSS, 10 mM Hepes, 0.1 % BSA, pH 7.4), in the presence of a range of concentrations of unlabelled MCP-1 or MCP-1–SEAP between 10 pM–200 nM. After incubation with the cells at 37 °C for 90 min, the plate was washed with HBSS, 10 mM Hepes, 0.5 M NaCl, pH 7.2, to remove any unbound ligand. The cells were then lysed by incubation at 37 °C for 2 h in a solution containing 1 % (w/v) SDS and 1 M NaOH, transferred to test tubes, and the radioactivity was measured (Clinigamma). Non-linear regression analysis of the data was performed using Prism 3.0 Software (GraphPad Software, San Diego, CA, U.S.A.).

### Interaction of MCP-1–SEAP and MCP-1 with HMEC-1 cells

A series of experiments was performed to assess the inhibition of MCP-1 binding to endothelial cells in the presence of heparin or chondroitin sulphate-C (CS-C; Sigma). In these assays, endothelial cells were cultured to confluence in 96-well plates and $^{125}$I-MCP-1 was added at a concentration of 100 pM in the presence of either heparin or CS-C (0–500 $\mu$g/ml). After incubation with the cells at 37 °C for 90 min, the plate was washed with binding buffer to remove any unbound ligand. Cells were then lysed and radioactivity was measured as described above. The amount of protein extracted from each well was assayed (bicinchoninic acid protein assay; Pierce Chemical Co., Perbio Science Ltd., Cheshire, U.K.), and the data were normalized to the percentage of maximal c.p.m. per mg of protein.

To assay MCP-1–SEAP binding to endothelial cells, HMEC-1 cells were grown to confluence in six-well clusters. The cells were washed with binding buffer and 100 nM MCP-1–SEAP was added in 0.6 ml of binding buffer in the absence or presence of either heparin or CS-C at a concentration of 250 $\mu$g/ml. Ligand binding was allowed to equilibrate at 37 °C for 90 min, after which the plate was washed with binding buffer to remove unbound MCP-1–SEAP. Bound MCP-1–SEAP was recovered by lysing the cells at 37 °C for 2 h in 0.5 ml of 10 mM Tris/HCl, pH 7.4, 1 % (v/v) Triton X-100 (Sigma). Samples were heat-denatured and SEAP assays were performed as above. The data were expressed as the percentage of maximal activity per mg of protein.

To assay competition between MCP-1 and the MCP-1–SEAP for binding to the surface of HMEC-1 endothelial cells, $^{125}$I-MCP-1, diluted to 100 pM in binding buffer, was added to cell monolayers in 96-well plates in the absence or presence of a 100-fold molar excess of MCP-1–SEAP. After incubation with the cells at 37 °C for 90 min, the plate was washed with binding buffer to remove any unbound ligand. Cells were then lysed, radioactivity was measured and the amount of protein extracted from each well determined as described above. Data were

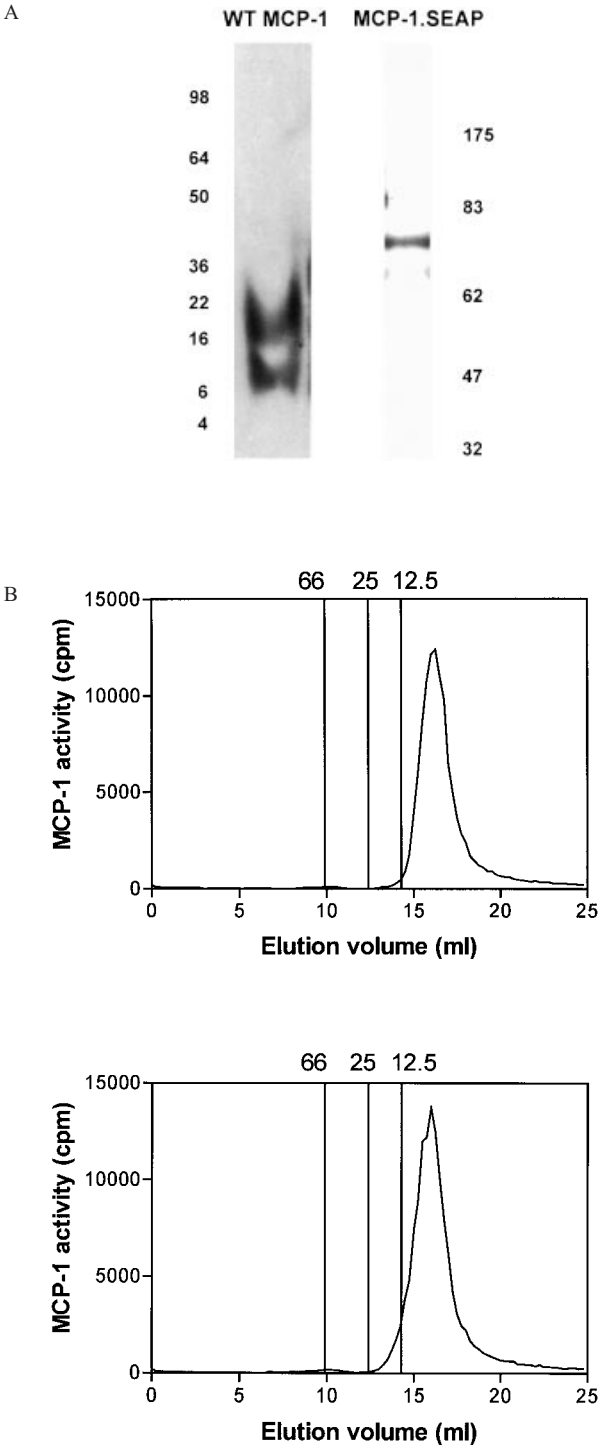


**Figure 1 MCP-1–SEAP behaves as a monomer under conditions which favour multimerization of native MCP-1**

(**A**) Western blot showing a single band of anti-MCP-1 reactivity in the MCP-1–SEAP fusion protein lane; this has an apparent molecular mass similar to that predicted for the intact construct. No additional bands with a higher molecular mass were observed. By contrast, the native protein (WT MCP-1) was clearly resolved as two bands with molecular masses corresponding to monomeric and dimeric forms of the protein. The position of molecular-mass markers (kDa) are indicated. (**B**) 30 nM MCP-1–SEAP was pre-incubated with 300 pM $^{125}$I-MCP-1 under physiological conditions prior to size-exclusion chromatography on a Superdex 75 column (upper panel). Results of same experiment with pre-addition of 500 $\mu$g/ml heparin (lower panel). The positions of the centre of elution peaks of molecular-mass standards are also shown with their masses in kDa indicated on upper axes.

expressed as the percentage of maximal c.p.m. per mg of protein.

### Measurement of $Ca^{2+}$ flux

The monocytic cell line THP-1 was used to model the mobilization of $Ca^{2+}$ following chemokine stimulation of mononuclear leucocytes. These cells were loaded in 1 ml of RPMI 1640 medium supplemented with 10 % FCS and 1 $\mu$M fura-2 (diluted from a 1 mg/ml stock in DMSO). After incubation for 30 min at 37 °C, the cells were washed and resuspended at $2 \times 10^6$ cells/ml in 140 mM NaCl, 4.5 mM KCl, 1 mM $MgCl_2$, 1 mM $NaH_2PO_4$, 2.5 mM $CaCl_2$, 5 mM glucose and 5 mM Hepes, pH 7.4. The cell suspension (1.5 ml) was placed in a fluorimetry cuvette and stirred continuously at 37 °C. The suspension was temperature-equilibrated for 10 min and then either native MCP-1 or MCP-1–SEAP was added at concentrations between 10–100 nM. Fluorescence was monitored every 1.5 s using excitation wavelengths of 340 and 380 nm and an emission wavelength of 510 nm on a PerkinElmer LS-5B luminescence spectrometer (PerkinElmer, Beaconsfield, Bucks, U.K.); the results were calculated as the ratio of emission following excitation at 340 nm with that produced by excitation at 380 nm. The maximum fluorescence was determined by treating loaded cells with 1 % (v/v) Triton X-100 and the minimum value measured after addition of 100 mM EGTA. The data were normalized as a percentage of the maximal fluorescence.

### Chemotaxis experiments

Peripheral blood mononuclear cells (PBMCs) were prepared from heparinized blood donated by healthy volunteers. Mononuclear cells were isolated by centrifugation at 400 ***g*** for 25 min at room temperature on a Lymphoprep (Amersham Pharmacia Biotech) density gradient according to the manufacturer's instructions. Harvested cells were rinsed twice and then resuspended in serum-free RPMI 1640 medium. Chemokine dilutions were prepared in the same medium. PBMCs were placed in the upper chamber of 3 $\mu$m pore size transwell chambers (Falcon; Becton Dickinson) on which a confluent monolayer of HMEC-1 endothelial cells had previously been seeded. The lower assay chamber contained serum-free RPMI 1640 medium. To generate dose–response curves, attractant was added to the lower assay chamber only. However, in some 'chequer-board' experiments, the chemokine was added to the upper, lower or both chambers, as required. For experiments designed to monitor the effect of addition of soluble GAGs, MCP-1 and MCP-1–SEAP were used at an optimum concentration of 12 nM and mixed with 250 $\mu$g/ml of either heparin or CS-C before addition to the lower chamber. After incubation for 3 h at 37 °C, the cells that had migrated to the lower chamber were recovered and stained with an R-phycoerythrin-conjugated anti-CD14 antibody (Dako, High Wycombe, U.K.). The number of CD14+ mononuclear phagocytes was determined by flow cytometric counting after addition of a standard number of FITC-labelled beads (Becton Dickinson).

Similar experiments were performed to measure chemotactic leucocyte migration across monolayer-cultured wild-type and 745-mutant CHO cells, following stimulation with 12 nM of either MCP-1 or the fusion protein. In addition, the effect of titrating heparin (10 ng/ml–10 $\mu$g/ml) with MCP-1 was measured in the assays using the 745-mutant CHO cells. The chemotactic peptide formyl-Met-Leu-Phe (10 nM; Sigma) was used as a positive control for the migration across wild-type and 745-mutant CHO cells. In separate experiments, it was shown

that the activity of this peptide was not modulated by the presence of heparin (results not shown).

## RESULTS

To investigate the biological properties of monomeric MCP-1, an MCP-1 fusion protein was produced. cDNA encoding human MCP-1 was cloned from the HMEC-1 microvascular endothelial cell line by reverse transcriptase-PCR. The identity and fidelity of the amplified product was confirmed by DNA sequencing. Using restriction sites introduced during amplification, MCP-1 was expressed with its C-terminus fused to the N-terminus of SEAP by a minimal RSSG linker. Following transfection of the NIH 3T3 cells, the stable cell clone MCPSEAP.14 was selected for expansion; medium conditioned by these cells regularly contained MCP-1–SEAP at a concentration in excess of 50 nM. Correlation of MCP-1 concentration in samples expressed from NIH 3T3 cells with SEAP activity indicated that the fusion protein had a specific activity of $0.165 \pm 0.008$ $\Delta A_{410}$/min per pmol.

To confirm that MCP-1–SEAP was monomeric under conditions that favour multimerization of the native protein, the two species were separated by SDS/PAGE and the results analysed by Western blotting. Figure 1(A) shows that two bands, corresponding to the predicted molecular masses of monomeric (8.6 kDa) and dimeric (17.2 kDa) forms of the native protein, were identified; faint bands, suggestive of higher-order species, were also observed on some gels. In contrast, the MCP-1–SEAP formed a single band with a molecular mass consistent with that predicted for the complete fusion protein (78 kDa); no higher-order species were observed.

In a further experiment, $^{125}$I-MCP-1 and MCP-1–SEAP were mixed at a 1:10 ratio and size-exclusion chromatography was performed. Figure 1(B, upper panel) shows that $^{125}$I-MCP-1 was eluted from a Superdex 75 column as a single monomeric peak with a molecular mass consistent with that of recombinant MCP-1 (8.6 kDa). Even after pretreatment of the mixture with 500 $\mu$g/ml soluble heparin, no higher-molecular-mass peaks, corresponding to mixed $^{125}$I-MCP-1 and MCP-1–SEAP oligomers (> 78 kDa), were observed (Figure 1B, lower panel).

Radioligand binding assays were performed to examine the interaction of both native MCP-1 and MCP-1–SEAP with CCR2b-expressing HEK-293 cells. Following a salt wash to disrupt non-specific ionic interaction with cell-surface GAGs, it was found that both native MCP-1 and the fusion protein competed for binding to the same cellular site with high affinity. Analysis of the competition curves showed that MCP-1 bound with an $IC_{50}$ of 0.75 nM, whereas the value for the MCP-1–SEAP fusion was 15 nM (Figure 2).

Further experiments were performed to investigate the binding of MCP-1 to cultured endothelial cells that express surface GAGs, but no specific receptors; in each of these assays, ligand binding was performed in the absence of a salt wash. Initially, it was found that titration of an increasing concentration of heparin, but not of CS-C, inhibited the binding of $^{125}$I-MCP-1

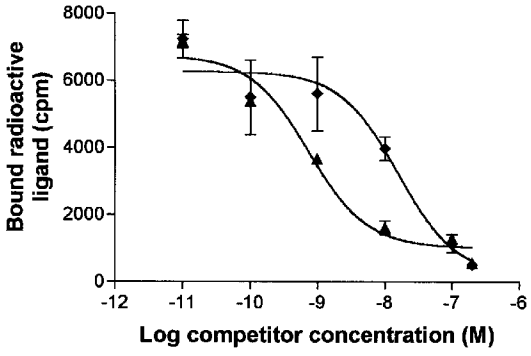


**Figure 2 Examination of ligand binding to the specific chemokine receptor CCR2b**

Competition binding curves showing changes in the amount of $^{125}$I-MCP-1 bound by HEK-293 cells expressing the CCR2b receptor during titration of MCP-1 (▲) or the MCP-1–SEAP fusion protein (◆). Data are means ± S.E.M. from a representative experiment ($n = 3$) repeated three times with similar results.

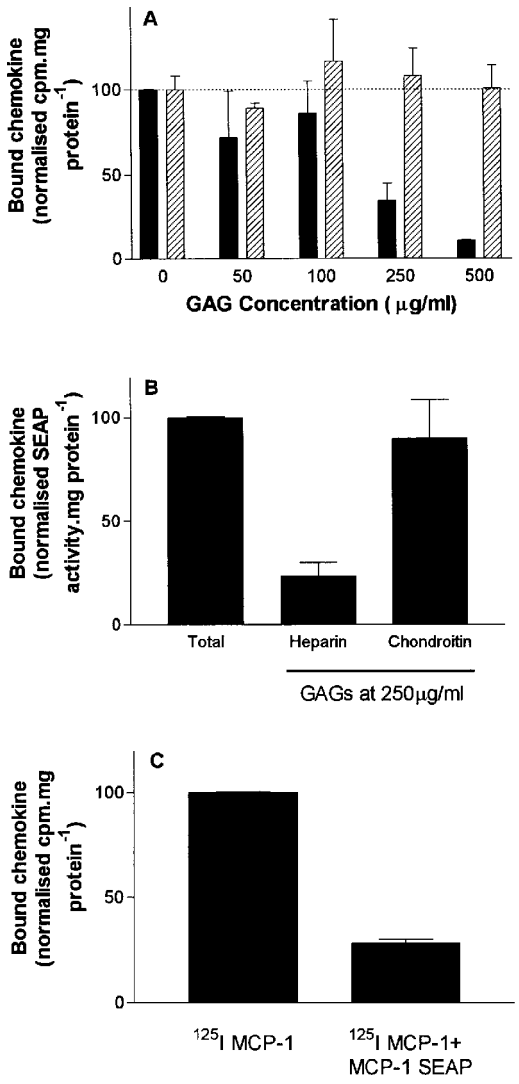


**Figure 3 Examination of MCP-1–SEAP and MCP-1 binding to the endothelial cell line, HMEC-1**

(**A**) Examination of the binding of $^{125}$I-MCP-1 to the surface of endothelial cells in the presence of a range of concentrations of heparin (solid bar) or CS-C (shaded bar). Data were normalized and expressed as means ± S.E.M. from a representative experiment ($n = 3$) repeated twice with similar results (**B**) Examination of the binding of MCP-1–SEAP to the surface of HMEC-1 endothelial cells in the presence of either heparin or CS-C at a concentration of 250 $\mu$g/ml. The experiment was performed three times with similar results; the data were normalized, with error bars showing the S.E.M. (**C**) Examination of the binding of $^{125}$I-MCP-1 to the surface of HMEC-1 endothelial cells in the presence of a 100-fold molar excess of MCP-1–SEAP. Data were normalized and expressed as means ± S.E.M. from a representative experiment ($n = 3$) repeated three times with similar results.

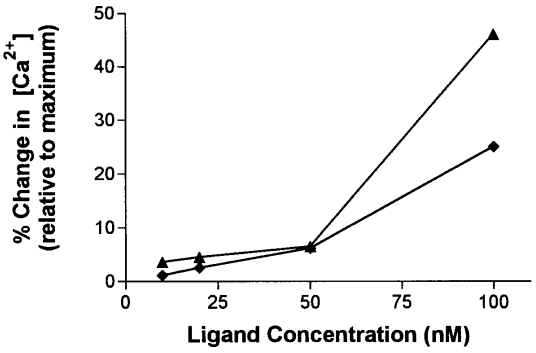


**Figure 4 MCP-1–SEAP binding induces signal transduction**

The change in intracellular $Ca^{2+}$ was measured following stimulation of the monocytic THP-1 cell line with a range of concentrations of both MCP-1 (▲) and the MCP-1–SEAP fusion protein (◆). The points represent the mean of duplicate determinations.

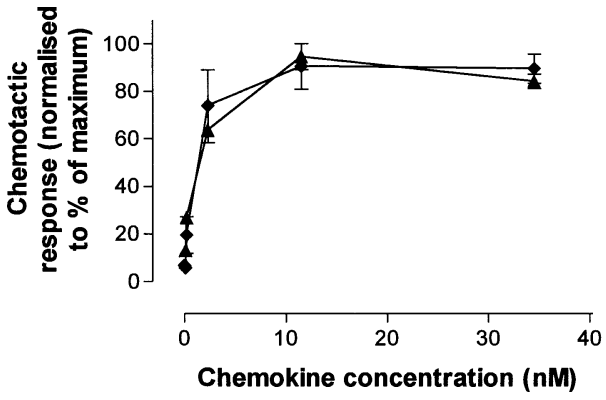


**Figure 5 The biological activities of MCP-1 and MCP-1–SEAP are indistinguishable**

The dose–response curve for trans-endothelial chemotaxis of CD14+ leucocytes towards MCP-1 (▲) and MCP-1–SEAP (◆). Data are means ± S.E.M. from a representative experiment ($n = 3$) repeated three times with similar results.

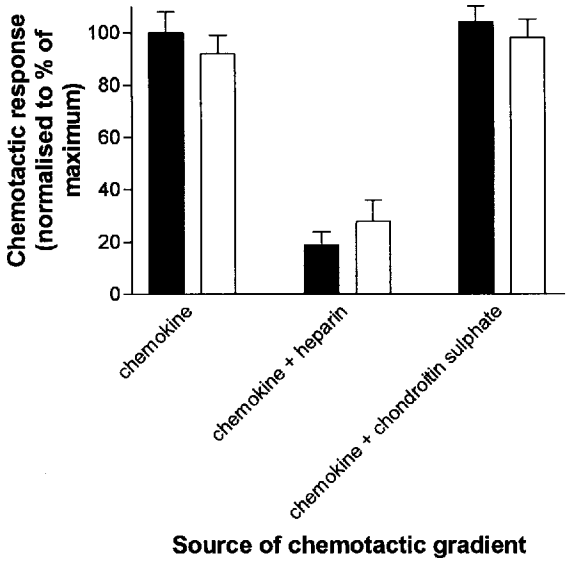


**Figure 6 The effect of pre-treatment of MCP-1 and MCP-1–SEAP with soluble GAGs on its capacity to induce leucocyte chemotaxis**

The chemotactic response promoted by 12 nM MCP-1 (solid bar) and the MCP-1–SEAP fusion (open bar) was compared with that observed after treatment with either 250 $\mu$g/ml heparin or CS-C. Results are means ± S.E.M. from a representative experiment ($n = 3$) repeated on three separate occasions with similar results.

to HMEC-1 cells; however, 30% of the ligand was bound by the cells at a heparin concentration of 250 $\mu$g/ml (Figure 3A). In a separate assay, it was found that the monomeric MCP-1–SEAP fusion protein showed a similar reduction in binding to endothelial cells in the presence of 250 $\mu$g/ml heparin (Figure 3B). The similarity observed between the binding of both iodinated native MCP-1 and the fusion protein was supported by the extensive competition between these species for binding to sites on cultured HMEC-1 endothelial cells (Figure 3C).

Following binding analysis, intracellular $Ca^{2+}$ mobilization was assayed using a leucocyte model system to demonstrate the biological activity of MCP-1–SEAP and confirm that the fusion protein mediated its effect through at least one of the receptor isotypes used by the native protein. In this series of experiments it was found that both native MCP-1 and the MCP-1–SEAP fusion were able to elicit a significant $Ca^{2+}$ flux in THP-1 cells (Figure 4).

Chemotactic migration assays were then performed to compare the ability of MCP-1 and MCP-1–SEAP to attract leucocytes across HMEC-1 endothelial cell monolayers. Initial 'chequerboard' experiments demonstrated that both the native and fusion protein could stimulate vectorial migration of a monocyte-enriched population of cells, indicating that both functioned as true chemotactic factors (results not shown). Detailed experiments were then performed to compare the dose–response profiles for the chemotaxis elicited by native and fusion chemokines (Figure 5). It was found that both MCP-1 and MCP-1–SEAP induced maximal leucocyte migration when added below the endothelial monolayer at a concentration of 12 nM.

To determine the significance of the interaction between GAG and either native MCP-1 or monomeric MCP-1-SEAP on chemotactic activity, the effect of mixing these species with soluble heparin or CS-C was examined. Figure 6 shows that 250 $\mu$g/ml heparin reduced the leucocyte migration elicited by both species to near-background levels, whereas a similar concentration of CS-C had no effect on the chemotactic response.

The potential of wild-type and 745-mutant CHO cells to support chemotactic migration was explored. The photomicrographs in Figure 7(A) show that both cell types adopt a similar morphology when grown to confluence on uncoated tissue-culture plastic. Furthermore, it was shown that the monocyte chemotactic response towards the non-heparin-sensitive chemoattractant formyl-Met-Leu-Phe was not significantly different ($P > 0.5$) across monolayers of wild-type and 745-mutant CHO cells (Figure 7B).

The role of cell-surface GAGs in supporting the chemotactic response towards MCP-1–SEAP was then examined by comparing leucocyte migration across monolayers of wild-type CHO cells with that across CHO cells expressing the GAG-deficient 745-mutation. Figure 8 shows that, in response to stimulation by MCP-1–SEAP, wild-type epithelial cells supported efficient leucocyte migration, although there was almost no chemotaxis across monolayers of the mutant cell line; a similar observation was made with native MCP-1. The failure of leucocyte migration across the 745-mutant CHO cell monolayers could not be overcome by the addition of a range of concentrations of soluble heparin.

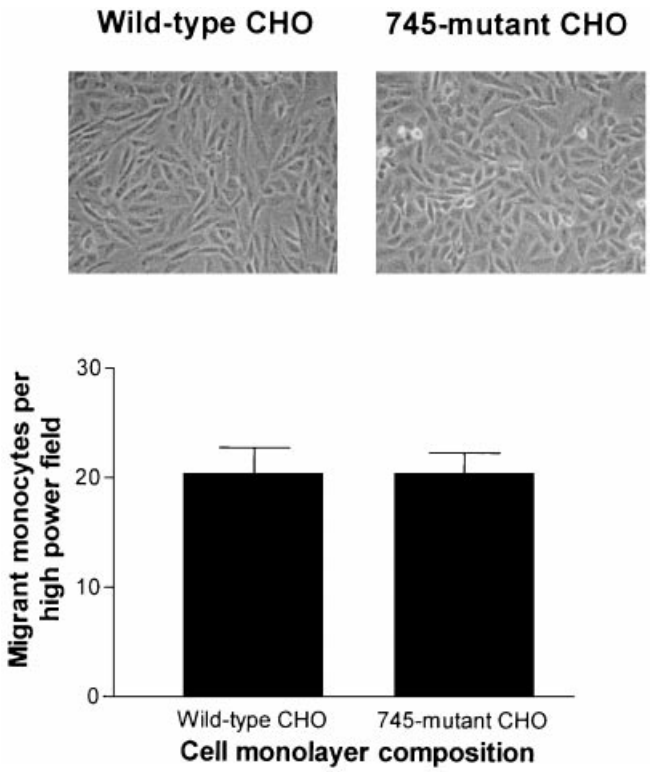


**Figure 7 Examination of chemotaxis across wild-type and 745-mutant CHO cells**

(**A**) Phase-contrast microscopic comparison of the morphology of monolayer cultured wild-type and 745-mutant CHO cells ( $\times$ 400). (**B**) Comparison of the potential of monolayers of both cell types to support monocyte chemotaxis towards a 10 nM solution of the non-heparin-sensitive chemoattractant, formyl-Met-Leu-Phe. The experiment was performed on 3 occasions with similar results. Chemotaxis was quantified for each CHO cell type by counting migrant cells in six high-power fields ( $\times$ 100) for each of three filters. Data are means $\pm$ S.E.M. from a representative experiment ($n = 3$) repeated on three occasions with similar results.

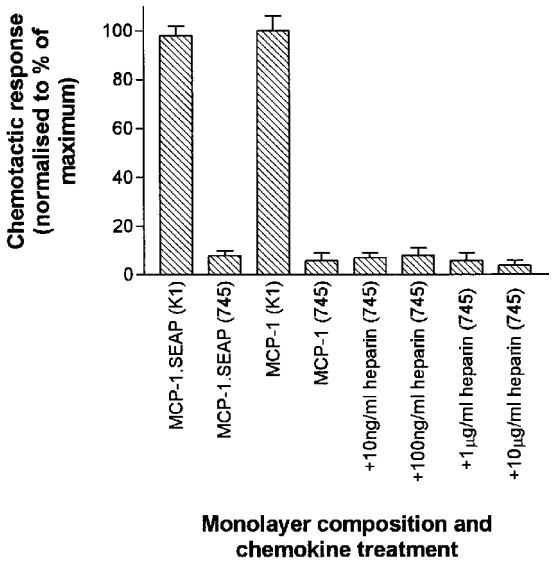


**Figure 8 The importance of GAGs for presentation of chemokines during leucocyte chemotaxis**

The number of CD14+ leucocytes induced to cross monolayers of wild-type CHO (K1) and GAG-deficient mutant CHO (745) cells in response to stimulation with 12 nM of either MCP-1 or the MCP-1–SEAP fusion protein was measured. Some of the MCP-1 assays on CHO 745 cells were supplemented with a range of concentrations of heparin. Results are means $\pm$ S.E.M. from a representative experiment ($n = 3$) repeated on three separate occasions with similar results.

## DISCUSSION

It has been reported that MCP-1 will form multimers in solution with an apparent $K_d$ of between 0.5–33 $\mu$M [17,21]. Indeed, a sensitive immunoprecipitation method has demonstrated the potential for at least partial multimerization of this chemokine at concentrations as low as 2.5 nM [15], which is clearly within a physiologically relevant range [20]. It is known that chemokines, including MCP-1, can bind GAGs such as heparan sulphate [25,26]; this interaction frequently includes specific peptide sequence domains such as BBXB (where B is a basic amino acid residue and X is any amino acid residue) [27]. It has been estimated that the concentration of heparin-like GAGs on the cell surface can reach 200 $\mu$g/ml [28]. The interaction with abundant GAGs could increase local chemokine concentrations [26], which might enhance multimerization. Indeed, Hoogewerf et al. [29] have used a variety of biochemical techniques to demonstrate the importance of heparin-like extracellular matrix components for promoting the self-aggregation of immobilized chemokines.

The functional significance of chemokine multimerization remains controversial, and might vary among chemokines. Zhang and Rollins [15] have used data from a cross-linking study to argue that the active form of MCP-1 is dimeric. However, Paavola et al. [17] engineered a mutant form of MCP-1, which lacked the capacity to dimerize, but retained a normal potential to bind and activate the specific CCR2b receptor. Similar work with interleukin-8 has demonstrated that a monomeric form is active [30,31], and monomeric MIP-1$\alpha$ mutants have equal activity compared with the native protein in monocyte shape-change assays [32].

It is clearly possible that multimerization might play an important role in regulation of MCP-1 function within the GAG-rich microenvironment surrounding the cell-surface receptor complex. To define the biological properties of native-sequence MCP-1 monomers, an MCP-1–SEAP fusion protein was produced in accordance with the methodology described by Luster et al. [23], who produced a SEAP fusion of the human CXC chemokine, IP-10. The SEAP moiety has a molecular mass of 69 kDa, and has been shown to prevent the aggregation of IP-10 by a mechanism that probably involves steric hindrance. Data from the current study clearly show that SEAP fusion can abrogate the potential of native MCP-1 to form stable oligomers. Although SEAP itself is functional as an easily dissociated dimer, the potential of this interaction to bring together and enhance dimerization of the MCP-1 component of the fusion protein is negated by emergence of the two N-termini on opposite surfaces of the dimeric alkaline phosphatase complex [33]. The SEAP-fusion technique is now used widely to provide a robust system to report gene expression [34], and has also been applied in non-radiochemical chemokine receptor binding studies [35].

In ligand binding experiments, MCP-1–SEAP was found to be an effective competitor of the binding of $^{125}$I-MCP-1 to cells expressing the CCR2b receptor. Following a salt wash to disrupt interaction with cell-surface GAGs, both native MCP-1 and the fusion protein bound specifically to the transfected cells with a high apparent affinity. The 20-fold decrease in receptor affinity

produced by fusion of MCP-1 with SEAP might be due to steric hindrance within the proteoglycan matrix, but still supports the contention that a monomeric form of MCP-1 is an effective ligand of the CCR2b receptor.

As endothelial cells do not express specific receptors for MCP-1, the sequestration of MCP-1 by cultured endothelial cells is suggestive of binding between the chemokine fusion protein and cell-surface GAGs. The capacity of soluble heparin to inhibit this interaction is indicative of competitive binding to domains on the chemokine molecule that are necessary for interaction with cell-surface heparan sulphate. The failure of matched concentrations of the structurally dissimilar GAG, CS-C, to inhibit binding to the endothelial cell surface is consistent with the findings of Kuschert et al. [26], who reported that CS-C is at least 20-fold less effective than heparin at inhibiting the binding of MCP-1 to cultured endothelial cells. As GAG chains are of variable lengths and can contain many protein binding domains, the stoichiometry for chemokine binding to GAG molecules is not 1:1. For this reason, comparison was made between the effect of mass-matched rather than molar concentrations of the GAG molecules.

The shallow competition curve produced by heparin titration is consistent with the existence of range of endothelial cell-surface GAG-protein binding sites of varying affinity. Even in the presence of soluble heparin at 250 $\mu$g/ml, some 30% of the iodinated ligand was bound by the endothelial cell surface; the failure of complete inhibition of ligand binding may be a reflection of the high concentration of heparan sulphate found within the proteoglycan layer on the surface of endothelial cells [28]. This result is similar to that observed for the MCP-1–SEAP fusion, and suggests that both native MCP-1 and the monomeric fusion protein interact with GAGs in the same way on the endothelial cell surface. This is supported further by the competition observed by the excess MCP-1–SEAP for cell-surface $^{125}$I-MCP-1 binding sites.

The MCP-1–SEAP fusion was engineered with the C-terminus of the chemokine fused to the N-terminus of the enzyme moiety. This avoided modification of the chemokine N-terminus, which has been shown to significantly alter the biological activity of $\beta$-chemokines [36–38]. The activity of the fusion protein was verified initially by examination of its potential to induce intracellular $Ca^{2+}$ mobilization in a monocytic cell line that is known to express appropriate CC chemokine receptors [39]. In this study, it was found that both native MCP-1 and MCP-1–SEAP were active, as both were able to elicit a $Ca^{2+}$-flux.

Using a simplified chequer-board assay, it was found that leucocyte migration across an endothelial cell monolayer was directed from a low to a higher concentration of either MCP-1 or MCP-1–SEAP. No significant migration occurred when equal chemokine concentrations were placed simultaneously in both the upper and lower assay chambers. These findings confirm that both MCP-1 and the monomeric fusion protein act as true chemotactic factors, rather than as promoters of migration by non-vectorial chemokinesis [11,40]. This observation is of importance for investigation of the biological activity of monomeric MCP-1, as chemotaxis can only occur in the presence of a stable chemotactic concentration gradient. It is most likely that this gradient is maintained *in vivo* by the sequestration of chemokines on to cell surface and matrix GAGs.

Further chemotaxis experiments demonstrated that both MCP-1 and MCP-1–SEAP were equally effective at promoting transendothelial leucocyte migration, with both species producing optimal migration when added to the basal chamber at 12 nM. This result indicates that multimerization within the cell surface or extracellular proteoglycan matrix is not necessary for the biological activity of MCP-1. This is consistent with the observation of Paavola et al. [17] that monomeric MCP-1 mutants were able to support chemotaxis within diffusion gradients across cell-monolayer-free polycarbonate membranes.

Despite the demonstration of the biological activity of monomeric MCP-1, it has been suggested that MCP-1 multimerization might facilitate binding to cell-surface GAGs and potentiate the formation of chemotactic concentration gradients [17]. In the present study, the importance of immobilization of the monomeric fusion protein by cell-surface GAGs was highlighted by the blockade of chemotaxis by soluble heparin, which can sequester MCP-1–SEAP from the cell surface. The similarity of this result to that observed for native MCP-1 is, again, consistent with both moieties interacting in the same way with cell-surface GAGs and soluble heparin, and strongly suggests that MCP-1 multimerization within the heparan-sulphate-rich cell-surface proteoglycan layer is irrelevant to the process of chemotactic extravasation.

Further evidence suggesting a relationship between chemokine sequestration and biological activity was provided by the demonstration that monolayers of GAG-deficient mutant cells, but not their wild-type counterparts, were unable to support a normal transmonolayer chemotactic response towards either native MCP-1 or the monomeric MCP-1–SEAP. Detailed study has shown that 745-mutant CHO cells have a specific deficiency in xylosyl transferase, resulting in under-expression of heparan sulphate [41]; for this reason these cells have been used extensively to examine the role of cell surface GAGs in protein binding [42]. It is known that the 745-mutant cells appear different from wild-type cells after plating on the heparin-binding substrate, fibronectin [43]. However, when plated on tissue-culture-treated plastic both wild-type and 745-mutant cells showed a similar morphology, an observation consistent with previous studies [41]. The equal chemotactic response towards the tripeptide, formyl-Met-Leu-Phe, observed across monolayers of wild-type and 745-mutant CHO cells provides clear evidence that both cell types are physically capable of supporting leucocyte migration. On this basis, it is most likely that the absence of chemotaxis across monolayers of heparan-sulphate-deficient CHO cells in response to MCP-1 is due to the failure of these cells to bind and present a stable transmonolayer concentration gradient of the chemokine.

Previous work has shown that a low concentration of soluble heparin can restore the potential of the heparin-binding molecule, basic fibroblast growth factor, to signal GAG-deficient indicator cells [42,44]. Significantly, the addition of soluble heparin did not restore a chemotactic response across monolayers of the GAG-deficient cells. A possible explanation for this is that soluble heparin–MCP-1 complexes fail to form a chemotactic gradient across monolayers of GAG-deficient cells. An alternative explanation is provided by a previous study from our group, which demonstrates that inappropriate interaction between chemokines and soluble heparin or heparan sulphate, but not CS-C, can reduce the potential for specific ligation of CC chemokine receptors [45].

The data in this report are consistent with a model in which MCP-1 is sequestered on to heparan sulphate molecules on the endothelial cell surface, for subsequent presentation to migrating leucocytes in the form of a solid-phase heparan sulphate bound chemotactic gradient [26,46]. Furthermore, it is clear that MCP-1 multimerization within the endothelial cell-surface proteoglycan matrix is not required for chemotactic activity.

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