Cutting Edge: Novel Human Dendritic Cell- and Monocyte-Attracting Chemokine-Like Protein Identified by Fold Recognition Methods

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Chemokines play an important role in the immune system by regulating cell trafficking in homeostasis and inflammation. In this study, we report the identification and characterization of a novel cytokine-like protein, DMC (dendritic cell and monocyte chemokine-like protein), which attracts dendritic cells and monocytes. The key to the identification of this putative new chemokine was the application of threading techniques to its uncharacterized sequence. Based on our studies, DMC is predicted to have an IL-8-like chemokine fold and to be structurally and functionally related to CXCL8 and CXCL14. Consistent with our predictions, DMC induces migration of monocytes and immature dendritic cells. Expression studies show that DMC is constitutively expressed in lung, suggesting a potential role for DMC in recruiting monocytes and dendritic cells from blood into lung parenchyma.

The role of chemokines has been best investigated in the immune system. They attract Ag-capturing and APC to tissues like skin and mucosal surfaces, which are primary sites of entry for pathogens to ensure immunosurveillance (3). To elicit an immune response after an infection with a pathogen, chemokines guide APC to secondary lymphoid organs, the foci meeting points of these cells with cells of the adaptive immune response, T and B cells. Furthermore, chemokines regulate homing of lymphocyte subtypes to subcompartments of lymphoid organs, a key function to ensure a coordinated immune response. Besides regulating cell trafficking, some chemokines have also been shown to play a role in processes such as angiogenesis and tumor growth (4, 5).

Many chemokines have been identified by their sequence signature motifs using sequence homology searches in databases (1). However, sequence homology-based methods fail as protein families become more diverse and remote homologues are difficult to identify below 20% sequence identity (6). Threading techniques utilize protein structural information to detect protein compatibility with known protein structures and, because they do not rely on sequence comparison, they are able to identify relationships even if sequence similarity is extremely low (7). In this study, we report the identification of a potential novel chemokine, DMC2 (dendritic cell (DC) and monocyte chemokine-like protein), by threading methods and its functional characterization.

Materials and Methods

Sequence identification and characterization

DMC (AY358433) sequence was previously identified (8) but sequence analysis (BLAST & Pfam) failed to identify any statistically significant sequence homology with any previously characterized protein.

Generating structure-based protein function hypothesis

The fold recognition algorithm ProHit (ProCeryon Biosciences) and a fold library consisting of 7950 representative three-dimensional (3D) protein structures from the Protein Data Bank were used. Sequence-structure alignments were generated with the Smith-Waterman algorithm (9). Two mean force potentials describing the energetic forces between the residues of a fold and between residues and the surrounding solvent were used to calculate the sequence-structure fitness (10, 11). Default ProHit values for gap restrictions were used to control the number, size, and placement of gaps in the query sequence and the fold library entries. The BLOSUM40 amino acid substitution matrix was used for sequence comparison and scoring (12). For ranking and scoring we used: 1) energy score derived from residue-residue and residue-solvent interactions (pair/surf), 2) sequence similarity, 3) a normalized combination of pair/surf and sequence similarity (threading index (Th. Idx.)), and 4) ratio between the fold...
length (fold length) and the number of aligned residues in the sequence-structure alignment (path length) (/flp). Results were ranked by their Th.Idx. /flp was used to exclude sequence-structure alignments not covering the full length corresponding to a specific fold and to rule out possible false positives. Based on previous studies (data not shown), hits with values $0.6 \approx /flp \approx 1.3$ were considered of a higher confidence. Corresponding 3D models were generated, and their respective sequence-structure alignments analyzed (ProHit and InsightII; Accelrys). The structural classification scheme SCOP (13) was used to build up a fold library containing all members of the IL-8-like fold family and to generate structure-based protein function hypothesis.

**DMC protein expression and purification**

His-tagged DMC was extracted from *Escherichia coli* inclusion bodies and purified on a Ni-NTA metal-chelate column. The Ni-NTA pool was applied onto a HiLoad 16/60 Superdex 75 gel filtration column (Amersham Biosciences) equilibrated with 20 mM MES (pH 6.0) containing 6 M guanidine HCl. Five milliliters of the Superdex 75 pool was treated with 50 mM DTT at pH 8.0, loaded onto a RP-HPLC Vydac C4 column equilibrated with 0.1% trifluoroacetic acid in water, and eluted with a gradient of acetonitrile (25–37%) in 0.1% trifluoroacetic acid. DMC was lophylized and dissolved in 1 mM HCl before dilution into assay buffer. A mutant form of DMC where all cysteines were converted to serines was generated. This His-tagged version was expressed and subjected to the same purification procedure as the wild-type protein.

**Baculovirus expression**

His-tagged DMC was cloned into the plasmid PH.HIF and transfected into SF29 cells using Lipofectin (Invitrogen Life Technologies). For screening of the Abs, ELISA plates (Immunoplate MaxiSorp; Nunc) were coated with 1 mg/ml His-tagged DMC. For screening of the Abs, ELISA plates (Immunoplate MaxiSorp; Nunc) were coated with 1 mg/ml His-tagged DMC. Anti-DMC mAbs were generated by immunizations of mice with recombinant baculovirus expression. His-tagged DMC was cloned into the plasmid PH.HIF and transfected into SF29 cells using Lipofectin (Invitrogen Life Technologies). After a 12-h culture in Hanks’ serum-free medium and 5 days in complete Hanks’ medium (Invitrogen Life Technologies), SF29 cells were infected with supernatant to generate a viral stock. A second amplification was done by infection of H9 cells in ESF21 medium (Expression System LLC) and DMC was purified using a Ni-NTA column.

**Circular dichroism**

DMC and CCL5 (R&D Systems) were dissolved in 1 mM HCl and diluted into PBS. Circular dichroism (CD) spectra were obtained in the far-UV range (190–290 nm) using quartz cuvettes of 1-mm path length (Aviv model 62DS CD spectrometer; Aviv Associates). DMC was measured at 0.5 mg/ml and CCL5 at 0.1 mg/ml. Data were collected at 2.0-nm intervals with bandwidth 1.0 nm and at 25°C.

**Cell isolation, culture, and treatments**

Human PBMC were isolated by Hypaque-Ficoll density centrifugation and cultured at 37°C in 5% CO2 at 10^6/ml in RPMI 1640, 10% FCS, 2 mM L-glutamine, 1 mM sodium pyruvate, penicillin, and streptomycin, both un-

**PBMC transmigrated across 5-µm Transwell migration filters (Corning) for 2.5 h in response to a stimulus in the bottom chamber and were enumerated by flow cytometry.**

**Immunohistochemistry**

Formalin-fixed, paraffin-embedded adult normal and inflamed lung, normal colon, and small intestine tissues were used for immunohistochemistry. Tissue sections were deparaffinized and hydrated. For Ag retrieval, slides were incubated in two rounds of Trilogy Ag retrieval solution (Cell Marque) at 99°C for 30 min. Endogenous peroxidase activity was quenched with 3% H2O2, then blocked with Vector Biotin Avidin Blocking reagents (Vector Laboratories), and the slides were blocked with 10% horse serum. Sections were stained with an in-house generated mouse anti-DMC mAb (clone 3H8) at 10 µg/ml, followed by a biotinylated horse anti-mouse Ab (Vector Laboratories) diluted 1/200 in blocking serum. For detection, Vector’s ABC kit was used and metal enhanced diaminobenzidine (Pierce). Sections were counterstained with Mayer’s hematoxylin.

**Results and Discussion**

**Assigning remote homologies**

DMC is a novel secreted protein of unknown function (8). It is a basic protein (pl ~10.9) of 119 aa and an estimated molecular mass of 13,819 Da. The putative signal peptide I cleavage site is between positions 23 and 24 (Fig. 1A) and it has no potential Asn-linked glycosylation sites. Its chromosomal location is 19:47,624,876–47,638,824.

Sequence similarity searches only identify a murine ortholog (71% identity; Fig. 1A). To search for structural homologies,
DMC’s sequence was threaded against a fold library of 7950 3D protein structures, obtaining a structural model for each fold. From the 20 top hits (Table I; see Materials and Methods for scoring and ranking), 9 were considered of “high confidence level,” of which only hits 6 and 12 were taken as “true positives” for not having high content of gaps, not lacking any secondary structure motif in the sequence-to-structure alignment, and presenting good matching of cysteines. Hits 6 and 12 correspond to IL-8-like chemokines (CCL3 and CXCL8, respectively). Based on these structural similarities, we assigned an IL-8-like fold to the DMC sequence. Secondary structure predictions performed for DMC (THD, DSC; data not shown) are in agreement with an IL-8-like fold. Src homology 2 (SH2)-like hits were considered false positives based on the fact that they had secondary structure elements missing in their alignments to DMC.

To assess accuracy of our structural hypothesis, we constructed a fold library containing all of the known IL-8-like structures (SCOP; data not shown). DMC and, as control, sequences of known IL-8-like proteins were threaded against this fold library, showing threading scores in the same range (sequence identity 8–16%; data not shown). The highest scores obtained for DMC were with a CXCL8 mutant (IL8E38C/C50A; sequence identity 8–16%; data not shown). The highest scores fold library, showing threading scores in the same range (sequences of known IL-8-like proteins were threaded against this structured a fold library containing all of the known IL-8-like elements to DMC.

They had secondary structure elements missing in their alignments to DMC. Furthermore, activation of monocytes and immature DCs (Fig. 2A). A similar migration activity was observed with a nontagged version of DMC (data not shown). To investigate whether DMC also recruits activated DCs and monocytes, PBMC were stimulated with LPS in cell culture before migration assays. LPS completely inhibited migration of DCs (Fig. 2A) and monocytes (data not shown) to DMC. Furthermore, activation of monocytes with PGE2 and forskolin significantly reduced the response to DMC (data not shown). Heat inactivation of DMC abolished migration of nonactivated DCs and monocytes (data not shown), as did mutation of all cysteines of DMC into serines (Fig. 2D).

The receptor to DMC is currently unknown, and there is no evidence that any of the known CXC receptors might also function as a receptor for DMC as tested in migration assays by addition of Abs to the various CXC receptors (data not shown). However, migration of DCs and monocytes was inhibited by DMC specifically induces migration of monocytes and DCs

To test whether human DMC shows chemotactic activity, we expressed his-tagged DMC in E. coli and performed Transwell migration assays with human PBMC. DMC specifically induced migration of CD14+ monocytes and CD14+ CD11c+ DCs (Fig. 2A). Other PBMC subtypes, such as CD3+ T cells, CD16+CD5+ D14+ neutrophils, and NK cells (Fig. 2A), or B cells (data not shown) were not attracted by DMC. As seen for other chemokines, the dose-response curve of monocyte and DC migration to DMC in the migration assay formed a bell-shaped response curve. Because proteins expressed in E. coli are not always folded correctly, we also expressed his-tagged DMC protein in the baculovirus system and confirmed the chemotaxis of monocytes and immature DCs (Fig. 2B). The receptor to DMC is currently unknown, and there is no evidence that any of the known CXC receptors might also function as a receptor for DMC as tested in migration assays by addition of Abs to the various CXC receptors (data not shown). However, migration of DCs and monocytes was inhibited by

### Table 1. Threading results and summary of structural and functional hypothesis

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| * | Percent sequence identity. |
| cl | Confidence level HC, high; FP, false positive. |
PTX (Fig. 2E), indicating that the receptor for DMC is a seven-transmembrane Goi protein-coupled receptor.

A panel of monoclonal mouse anti-DMC Abs was generated and selected by binding to DMC but not to the his tag (Fig. 3A). A subset of the Abs was further characterized, and several Abs were found to completely inhibit migration of monocytes and DCs as shown for clone 3H8 (Fig. 3B). Clone 3H8 is an IgG1 Ab with a L chain. It also recognized DMC specifically by Western blot (data not shown). Anti-CCL2 Abs did not block migration to DMC (Fig. 3B). Furthermore, DMC protein bound specifically to monocytes and DCs but not to T cells as detected by flow cytometry using the monoclonal anti-DMC Ab and freshly isolated PBMC (data not shown).

The majority of other monocyte-attracting chemokines preferentially induce migration of activated monocytes (16), CCL2 is specific for attraction of activated monocytes to sites of inflammation (17), and CXCL14 preferentially attracts monocytes activated with PGE2, or forskolin (18). CX3CL1, which is expressed by bronchiolar epithelial cells in chronic granulomatous inflammation of the lung, attracts subsets of monocytes (19).

Circulating blood DCs express a variety of chemokine receptors, CCR1, CCR2, CCR3, CCR5, and CXCR4 (20). They have been shown to respond to the CC chemokines CCL2, CCL8, CCL13, CCL5, and CCL11, which allows their recruitment to sites of inflammation (20). Migration of blood DCs to noninflammatory sites may be regulated by CXCL12, an ubiquitously expressed chemokine (21). CCL20, a chemokine expressed in noninflamed lung and liver, may recruit certain subsets of immature DCs into these tissues (22). However, unlike immature DCs generated in vitro from CD34+ bone marrow cells, blood DCs do not express CCR6 and therefore do not respond to CCL20 (23). We propose that DMC may fulfill this role.

DMC expression

Northern blot analysis using human multitissue blots showed that DMC is expressed in adult trachea, stomach (Fig. 4A), and fetal lung (Fig. 4B). Immunohistochemistry analysis of adult normal lung tissue sections demonstrated that DMC is constitutively expressed on bronchial and bronchiolar epithelium (Fig. 4C), as well as in a subset of alveolar lining cells (Fig. 4E). In addition, DMC expression was also observed in lung tissue from patients with asthma or obstructive pulmonary disease (data not shown). The expression levels and pattern were similar regardless of the inflammation status of the lung, suggesting that chronic inflammatory processes do not regulate DMC expression. However, acute inflammatory conditions have not been investigated yet. Furthermore, DMC expression was also observed in adult, normal small intestine (duodenum) and colon tissue sections (Fig. 4G and I). Specifically, DMC staining is detected in the villus and some crypt epithelial cells of the small intestine (Fig. 4G) and in colonic epithelial cells, primarily at the luminal side (Fig. 4I). Constitutive expression of DMC in lung, stomach, colon, and small intestine supports a
potential role for DMC as a housekeeping chemokine regulating recruitment of nonactivated blood monocytes and immature DCs into tissues. The presence of APCs at mucosal surfaces of these tissues is of biological importance for immunosurveillance of potentially harmful pathogens that may enter the lungs with air intake and the intestinal tract via food intake. If a pathogen invades, it will quickly activate the local APCs. Consequently, the local DCs mature, stop responding to DMC, and migrate to secondary lymphoid organs, where they activate the adaptive immune response to eradicate the pathogen.

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Disclosures

The authors have no financial conflict of interest.

References


FIGURE 4. Expression pattern of DMC in normal human tissues. Northern blot analysis of total RNA from adult (A) and fetal (B) human tissues probed to detect DMC. C–F, Expression of DMC in adult human lung. IHC analysis of normal lung sections stained with an anti-DMC Ab (C and E) or an isotype control Ab (D and F). DMC is detected in human bronchial epithelium (BE) and a subset of alveolar lining cells (ALC). Stainings are representative of 26 lung samples from patients with asthma, chronic obstructive pulmonary disease, or normal lungs. G–J, Expression of DMC in adult human small intestine (duodenum) and colon. Sections were stained with an anti-DMC Ab (G and I) or an isotype control Ab (H and J). DMC is detected in small intestine villus and some crypt epithelial cells (G). DMC is also detected in colonic epithelial cells (I), primarily at the luminal surface. Stainings are representative of three normal patients for each tissue type.