Macrophage-derived Chemokine Is a Functional Ligand for the CC Chemokine Receptor 4

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Macrophage-derived chemokine (MDC) is a recently identified member of the CC chemokine family. MDC is not closely related to other chemokines, sharing most similarity with thymus- and activation-regulated chemokine (TARC), which contains 37% identical amino acids. Both chemokines are highly expressed in the thymus, with little expression seen in other tissues. In addition, the genes for MDC and TARC are encoded by human chromosome 16. To explore this relationship in greater detail, we have more precisely localized the MDC gene to chromosome 16q13, the same position reported for the TARC gene. We have also examined the interaction of MDC with CC chemokine receptor 4 (CCR4), recently shown to be a receptor for TARC. Using a fusion protein of MDC with secreted alkaline phosphatase, we observed high affinity binding of MDC-secreted alkaline phosphatase to CCR4-transfected L1.2 cells ($K_d = 0.18$ nM). MDC and TARC competed for binding to CCR4, while no binding competition was observed for six other chemokines (MCP-1, MCP-3, MCP-4, RANTES, regulated on activation normal T cell expressed and secreted), macrophage inflammatory protein-1α, macrophage inflammatory protein-1β). MDC was tested for calcium mobilization in L1.2 cells transfected with seven different CC chemokine receptors. MDC induced a calcium flux in CCR4-transfected cells, but other receptors did not respond to MDC. TARC, which also induced calcium mobilization in CCR4 transfectants, was unable to desensitize the response to MDC. In contrast, MDC fully desensitized a subsequent response to TARC. Both MDC and TARC functioned as chemokacters for CCR4 transfectants, confirming that MDC is also a functional ligand for CCR4. Since MDC and TARC are both expressed in the thymus, one role for these chemokines may be to attract CCR4-bearing thymocytes in the process of T cell education and differentiation.

Chemokines are small secreted proteins that mediate recruitment of leukocytes to sites of inflammation (1, 2). The complexity and functions of the chemokine family have become increasingly diverse as more members have been identified and characterized. There are four subfamilies of chemokines based on the relative position of conserved cysteine residues (1, 3, 4).

The largest subfamily consists of the CC chemokines, which generally induce migration of monocytes, T lymphocytes, and in some cases eosinophils, basophils, or mast cells. All chemokines mediate their activities through G protein-coupled receptors, which have a characteristic seven-transmembrane structure. These receptors are very selective and bind specific ligands with high affinity. Eight different CC chemokine receptors have been characterized to date, CCR1, CCR2, CCR3, and CCR5 each has ligand specificity for at least three CC chemokines (5–12), while single high affinity ligands have been identified that recognize CCR4, CCR6, CCR7, and CCR8 (13–17).

Macrophage-derived chemokine (MDC) is a novel CC chemokine synthesized by macrophages and dendritic cells (18). MDC shares only limited homology with other chemokines. It is most similar to thymus- and activation-regulated chemokine (TARC) (19), with 37% identity. As shown by tissue Northern blots, MDC also shares a very similar expression pattern with TARC, showing high levels in thymus and very low expression in other tissues. Most surprisingly, MDC and TARC are encoded by human chromosome 16 (18, 20), while other CC chemokines are closely linked on chromosome 17 (21).

TARC was recently found to be a highly specific ligand for CCR4 (13), and both are co-expressed in the thymus (19, 22). TARC is likely to be made by dendritic cells and may aid the recruitment, activation, and development of T cells that express CCR4. Because of the similarities between MDC and TARC, a study was undertaken to determine if MDC interacts with CCR4, the receptor for TARC. We show here that MDC is a ligand for CCR4 and in fact binds with a higher affinity than TARC.

EXPERIMENTAL PROCEDURES

Chromosomal Localization—A 20-kilobase pair genomic fragment containing the human MDC gene was labeled with digoxigenin by nick translation and used as a probe for fluorescence in situ hybridization of human chromosomes (Genome Systems, Inc., St. Louis, MO). The labeled probe was hybridized to normal metaphase chromosomes derived from phytohemagglutinin-stimulated peripheral blood lymphocytes. Reactions were carried out in the presence of sheared human DNA in 50% formamide, 10% dextran sulfate, 30 mM sodium chloride, 3 mM sodium citrate, and 0.1% sodium dodecyl sulfate. Hybridization signals were detected by treating slides with fluoresceinated antidigoxigenin antibodies followed by counterstaining with 4,6-diamidino-2-phenylindole. Initial labeling implicated a group E chromosome. A genomic probe that specifically hybridizes to the short arm of chromosome 16 was used to demonstrate cohybridization of chromosome 16 with the MDC probe. A total of 80 metaphase cells were analyzed, with 61...
Cell Culture—The murine pre-B cell line L1.2 was kindly provided by Dr. Craig Gerard (Harvard Medical School, Boston, MA) and maintained in RPMI 1640 medium supplemented with 10% fetal bovine serum. L1.2 cells were stably transfected as described previously (13) by electroporation with 10 \( \mu \)g of linearized plasmid at 260 V, 960 microfarads using a Gene Pulser (Bio-Rad). HUT78 cells (ATCC) were maintained in Iscove's modified Dulbecco's medium with 15% fetal bovine serum.

Preparation of Recombinant Chemokines—The mature sequences of both MDC and TARC were chemically synthesized by Gryphon Sciences (South San Francisco, CA) using \( t \)-butyl-oxycarbonyl chemistries on a

FIG. 1. Localization of human MDC to chromosome 16. Fluorescent in situ hybridization of human metaphase chromosomes to the MDC genomic DNA probe demonstrates hybridization to 16q13 (arrows). Chromosomal identification was confirmed with a chromosome 16-specific probe (not shown). The idiom on the right illustrates the chromosomal position of the MDC hybridization to 16q13.

FIG. 2. Binding characteristics of MDC-SEAP to CCR4. A, saturable binding of MDC-SEAP to L1.2 cells expressing CCR4. Cells were incubated with increasing concentrations of MDC-SEAP as described under “Experimental Procedures.” Cells were washed four times in binding buffer, and the amount of bound MDC-SEAP was determined enzymatically. Nonspecific binding was determined by the addition of a 200-fold molar excess of unlabeled MDC. B, Scatchard analysis of the binding data in panel A. The y axis (B/F) represents [bound]/[free]. C, displacement of the binding of MDC-SEAP to CCR4-transfected L1.2 cells by unlabeled MDC and TARC. Cells were incubated with 0.5 nM MDC-SEAP in the presence of the indicated concentrations of unlabeled MDC or TARC. The calculated IC \( _{50} \) is 0.18 nM for MDC and 0.62 nM for TARC. D, displacement of MDC-SEAP by other chemokines. L1.2 cells stably transfected with human CCR-4 were incubated with 0.5 nM MDC-SEAP in the presence of 100 nM of the indicated chemokines. Cells were washed, and specifically bound MDC-SEAP was determined enzymatically.
peptide synthesizer (model 430A; Applied Biosystems). Lyophilized protein was dissolved at 10 mg/ml in 4 mM HCl and immediately diluted to 0.1 mg/ml in phosphate-buffered saline plus 0.1% bovine serum albumin (BSA) for storage at −80 °C. Recombinant MDC was expressed as a fusion protein with the secreted form of plasmid alkaline phosphatase (SEAP) as described (13), in the expression vector pcDNA3 (CLONTECH, Palo Alto CA). Briefly, the coding region of MDC followed by the coding sequence for a five-amino acid linker sequence (Ser-Arg-Ser-Ser-Gly) was fused in frame to the mature coding region of SEAP. The expression plasmid was transfected into COS cells by the method of Targowski and Kamen (20). For cell stimulation, the serum levels were reduced from 10 to 1%. After 3–4 days, the MDC-SEAP expression plasmid was transfected into COS cells by the calcium phosphate precipitate method with a calcium phosphate precipitate:DNA ratio of 1:1 (11). The transfected cells were then stimulated with 10 nM MDC for 16 h. The concentration of MDC-SEAP was determined by comparison with the reported specific activity (22). After washing twice, cells were resuspended at 2.5 × 10^6 cells/ml. To measure intracellular calcium, cells in 2 ml were placed in a quartz cuvette in a Perkin-Elmer LS 50B spectrophotometer. Fluorescence was monitored at 340 nm (excitation wavelength 1), 380 nm (excitation wavelength 2), and 510 nm (emission wavelength) every 200 ms. Chemotaxis Assay—Cell migration was assayed using L1.2 cells transfected with CCR4 cDNA (13) or HUT78 cells. Approximately 10^6 cells resuspended in 0.1 ml of RPMI 1640 medium with 0.5% BSA were loaded in the upper wells of a transwell chamber (3-µm pore size, Costar). Test chemokines were added to the lower wells in a volume of 0.6 ml. After 4 h at 37 °C, cells in the lower chamber were collected and counted by fluorescence-activated cell sorting. Values are expressed as the percentage of input cells that migrate through the filter. Untransfected L1.2 cells were used as a control.

Northern Analysis—The expression of CCR4 mRNA in T cells was examined by Northern analysis. Total RNA was extracted from the T cell line HUT78 using RNA Stat-60 (Tel-Test "B", Friendswood, TX). Peripheral blood lymphocytes (PBL) were obtained from human blood separated over Histopaque gradients. After separation, monocytes were removed by plastic adherence. Half of the cells were used immediately to obtain RNA ("unstimulated") and the other half were treated with IL-2 (50 units/ml, Boehringer Mannheim) for 10 days before extracting RNA. Ten µg of total RNA was loaded per lane, fractionated on 0.8% agarose-formaldehyde gel, and transferred to nitrocellulose as described (12). The blot was probed with a gel-purified polymerase chain reaction fragment containing the entire coding region of CCR4 (22) and washed as described (12).

RESULTS

Chromosomal Localization—To more precisely establish the chromosomal location of the human MDC gene, fluorescent in situ hybridization analysis was performed using a 20-kilobase pair human genomic clone as a probe. An initial hybridization experiment localized the gene to the q terminus of a group E chromosome. Subsequent co-hybridization experiments with a genomic probe specific for chromosome 16 implicated the q terminus of chromosome 16. The MDC probe hybridized to a region immediately adjacent to the heterochromatic/euchromatic boundary, corresponding to band 16q13. This localization is depicted in Fig. 1. Because the TARC gene is localized in this region (20), we next compared the receptor usage of MDC with TARC.
Receptor Binding Assays—Chemokines have previously been fused to SEAP to generate probes for receptor binding studies (20, 25). We utilized this strategy to prepare MDC as a fusion protein with SEAP. A plasmid was prepared that directed the expression of a secreted fusion protein with MDC at the amino terminus followed by the alkaline phosphatase with His6 tag. The MDC-SEAP was used as a probe to examine binding to CCR4-transfected L1.2 cells. As shown in Fig. 2A, the MDC-SEAP bound to CCR4-expressing cells in a saturable manner. This binding was to a single high affinity site with a $K_d$ of 0.18 nM, as demonstrated by Scatchard analysis (Fig. 2B). Binding of MDC-SEAP was competitively inhibited with increasing concentrations of unlabeled MDC or TARC (Fig. 2C). The IC50 for MDC was 0.65 nM, while the IC50 for TARC was 2.1 nM. This suggests that both ligands recognize a common binding site on CCR4 and that MDC has more than 3-fold higher affinity for CCR4. To examine specificity of binding to CCR4, six additional chemokines were tested for competition of MDC-SEAP binding. A 200-fold molar excess of each chemokine was tested for competition with a constant quantity of MDC-SEAP (0.5 nM), presented in Fig. 2D. The chemokines MCP-1, MCP-3, MCP-4, RANTES, MIP-1$, and MIP-1$ did not compete for binding of MDC-SEAP to CCR4. In contrast, both MDC and TARC blocked binding to CCR4 transfectants.

MDC Induces Calcium Flux in CCR4-transfected Cells—TARC has previously been shown to signal through CCR4 by inducing calcium mobilization (13). To determine the ability of MDC to cause signaling through chemokine receptors, we examined calcium mobilization in L1.2 cells expressing CCR1, CCR2B, CCR3, CCR4, CCR5, CCR6, or CCR7 (Fig. 3). MDC did not cause calcium flux in L1.2 cells transfected with CCR1, CCR2B, CCR3, CCR5, CCR6, or CCR7, whereas each responded to its known cognate ligand. In contrast, L1.2 cells transfected with CCR4 produced a strong calcium flux when stimulated with 10 nM MDC. Similar to other G protein-coupled receptors, CCR4 was refractory to subsequent stimulation with the same concentration of MDC. MDC also completely desensitized CCR4 transfectants to subsequent TARC treatment when both were added at 10 nM. However, pretreatment with TARC did not desensitize the receptor to subsequent stimulation with MDC. The signal produced by initial TARC stimulation was of lower intensity than the primary MDC signal and the MDC signal secondary to TARC stimulation. These results further confirm that MDC is a ligand for CCR4.

MDC Induces Chemotaxis in Cells with CCR4—We next examined the ability of MDC to induce migration of CCR4-transfected L1.2 cells. As shown in Fig. 4A, both MDC and TARC induced migration of CCR4-transfected L1.2 cells. Both chemokines produced classic bell-shaped migration responses with maximal migration between 1 and 10 nM. The migration for MDC was significantly higher than that for TARC. Untransfected L1.2 cells failed to migrate when treated with MDC. These chemotaxis results confirm that both MDC and TARC are functional ligands for CCR4. The human T cell line HUT78 has previously been shown to express CCR4 mRNA and to respond to TARC (13). As shown in Fig. 4B, both MDC and TARC induced migration of this T cell line.
Binding of MDC was also examined on HUT78 cells. The MDC-SEAP fusion bound to the HUT78 T cell line with high affinity and could be competed with a 200-fold molar excess of MDC, as presented in Fig. 5A. A Northern blot analysis, Fig. 5B, confirms the expression of CCR4 in HUT78 cells and PBL. The expression of CCR4 is greatly increased when PBL are stimulated with IL-2. These experiments suggest that the MDC activation of CCR4 seen on transfectants and the HUT78 T cell line are likely to also occur on natural T cells.

**DISCUSSION**

As demonstrated by binding studies, calcium mobilization, and chemotaxis, MDC is a potent agonist for CCR4. The high affinity binding and concentrations of MDC required for signaling are similar to other chemokine receptor-ligand interactions. MDC and TARC both bind and signal through CCR4. In addition to this similar activity, both chemokines share similar in vivo expression patterns and are closely linked on human chromosome 16. MDC and TARC share these functional similarities despite sharing only 37% amino acid identity. Consequently, MDC and TARC can be considered to comprise a distinct class of CC chemokines with unique genetic localization, receptor utilization, expression pattern, and function.

Our experiments suggest that MDC is likely to act as a chemoattractant for CCR4-expressing cells including mature T lymphocytes. This prediction has been confirmed recently by other investigators. Chang and colleagues (26) have shown that MDC acts as a chemoattractant for activated T cells. Pal and colleagues (27) have demonstrated that MDC induces calcium mobilization in activated T cells and also acts as an human chemokine receptor-ligand interaction, receptor utilization, expression pattern, and function.

During T cell development, immature progenitor cells undergo differentiation and expansion leading to the establishment of the major T cell lineages and the elimination of potentially autoreactive T cells (28). These processes occur within the highly specialized microenvironment of the thymus. The signals regulating the directed movement of immature T cells within the thymus have yet to be determined. The abundant expression of MDC, TARC, and CCR4 in the thymus (with very little expression seen in other tissues (18, 19, 22)) suggests that they may play a role in T cell development. CCR4 is expressed on T cells (13, 22), while MDC and TARC are expressed by cells that may play a role in T cell development. CCR4 is expressed on T cells (13, 22), while MDC and TARC are expressed by cells of the dendritic lineage, which form a major component of the thymic architecture (18, 19). MDC and TARC may function to attract or retain T cells in the thymus and thereby mediate their trafficking and education. To further elucidate the physiologic role of these molecules, responses on T cell subsets should be determined. The complexity of the cellular movements that need to occur during T cell development may explain the diversity of chemokine and chemokine receptor expression within the thymus. Other chemokines such as PARC (pulmonary and activation-regulated chemokine; Ref. 29/DC-CK1 (30), SCM-1 (31)/lymphotactin (3), and TECK (thymus-expressed chemokine; Ref. 32) and receptors such as CCR5 (16, 17, 33) exhibit the greatest expression in the thymus. Determining the relationships and functions of these molecules may greatly help to understand the mechanisms of T cell development and regulation.

MDC may also play a role outside of the thymus in certain disease states. We have previously shown that MDC is expressed at high levels by cultured macrophages and dendritic cells (18). CCR4 is also expressed at high levels by activated T lymphocytes, principally of the CD4 subset (13). Thus, MDC may also play a role in the initiation and or triggering of the immune response by facilitating the interaction of T cells with antigen-presenting cells at sites of inflammation.

MDC was previously shown to stimulate migration of dendritic cells and IL-2 activated natural killer cells (18). It is not clear if this migration is mediated by CCR4, since it appears to be expressed primarily on T cells. MDC may be recognized by other chemokine receptors that are yet to be characterized. Further experiments are required to determine if TARC is also able to stimulate dendritic cell migration. By virtue of its ability to attract both T lymphocytes and dendritic cells, MDC may play a unique role in the initiation or amplification of antigen-specific immune responses.

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**REFERENCES**