Brief Definitive Report

IDENTIFICATION AND CHARACTERIZATION OF SPECIFIC RECEPTORS FOR MONOCYTE-DERIVED NEUTROPHIL CHEMOTACTIC FACTOR (MDNCF) ON HUMAN NEUTROPHILS

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A novel human monocyte-derived neutrophil chemotactic factor (MDNCF), which is clearly distinct from either IL-1 or TNF, has been identified and purified from LPS-stimulated human monocyte-conditioned media. MDNCF has been shown to be a specific chemoattractant for neutrophils but not for monocytes (1, 2). The cDNA for MDNCF has been cloned and the amino acid sequence of the protein has been deduced (3). The mature form of MDNCF consists of 72 amino acids. An identical and fully active MDNCF has been chemically synthesized (4) and expressed in *Escherichia coli* (R. Furuta, M. Yamada, and K. Matsushima, manuscript submitted for publication).

MDNCF has the capability to chemoattract neutrophils and also stimulate degranulation and respiratory burst response, resulting in release of lysosomal enzymes and generation of superoxide anion and hydrogen peroxide (5, 6). The directional migration of neutrophils in response to very low concentrations of MDNCF suggests the presence of specific plasma membrane receptors on neutrophils through which the activating peptide exerts its effects. The recent availability of a large amount of pure, active recombinant MDNCF has permitted us to examine whether polymorphonuclear neutrophils (PMN) express specific cell surface receptors for MDNCF.

Materials and Methods

Reagents. Human rIL-1α (2×10^7 U/mg) and TNF-α (10^7 U/mg) were obtained from Dainippon Pharmaceutical Company, Osaka, Japan. Recombinant human MDNCF (2×10^6 U/mg) has been expressed in *E. coli* and purified to homogeneity (R. Furuta, M. Yamada, and K. Matsushima, manuscript submitted for publication). C5a, LTB4, PAF, CHAPS, DSS, and protease inhibitors were purchased from Sigma Chemical Co., St. Louis, MO.

Preparation of Human Neutrophils. Citrated peripheral venous blood was obtained from normal human volunteers and a granulocyte-enriched fraction was obtained by leukapheresis from the National Institutes of Health Clinical Center Transfusion Medicine Department, Bethesda, MD. Neutrophils were purified as described by English and Andersen (7). 95–98% of cells were identified as PMN and 2–5% cells were mononuclear cells with Wright's stain. The human promyelocytic cell line HL60 (8) was maintained and cultured in RPMI 1640 supplement with 10% FCS.

Radiolabeling of Human MDNCF. To radiolabel human MDNCF, 20 μg of pure recom-

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binant material in 50 μl 0.1 M borate buffer, pH 8.5, was added to 1 mCi ^{125}I-labeled (2,200 Ci/mmol; monoiodinated) Bolton Hunter reagent, incubated, and fractionated as described (9). The biological potency of the radiolabeled material was checked by assaying myeloperoxidase release from neutrophils as described by Schroder et al. (5).

Binding of ^{125}I-MDNCF to Neutrophils. Binding of the labeled MDNCF to the intact human neutrophils was carried out as previously described for IL-1 (9). In standard binding assays, 2×10^6 cells were incubated in duplicate with 4 ng ^{125}I-MDNCF in RPMI 1640 medium with 10 mg/ml BSA in a total volume 200 μl. The nonspecific binding was carried out by parallel incubation in the presence of 80-fold excess of unlabeled MDNCF.

Chemical Crosslinking and SDS-PAGE Analysis. For chemical crosslinking experiments, 10^7 neutrophils suspended in 1 ml RPMI 1640 medium were incubated with ^{125}I-MDNCF (20 ng) for 1 h at 4°C. Parallel experiments were performed in the presence of unlabeled MDNCF (2,000 ng) in a similar manner. After incubation, the cells were washed twice with 1 ml cold D-PBS and were suspended in 100 μl D-PBS. Then, disuccinimidyl suberate (DSS) at varying concentrations in DMSO was added to give a final concentration of 0.75, 1, and 1.25 mg DSS/ml. The mixtures were incubated for 1 h at 4°C. The cells were washed with cold D-PBS twice and suspended in 50 μl D-PBS containing 9 mM CHAPS and 2 μl of the mixture, each containing PMSF (12.8 ng), leupeptin (16 ng), pepstatin (80 ng), and chymostatin (2 ng). The detergent extraction mixtures were incubated on ice for 5 min and then centrifuged at 10,000 *g* for 15 min at 4°C. SDS-PAGE was done as described by Laemmli (10), followed by autoradiography.

Results and Discussion

Radiolabeling of MDNCF. Carrier-free recombinant MDNCF was labeled with Bolton Hunter reagent and chromatographed on a Sephadex column. The radiolabeled MDNCF was homogenous on SDS-PAGE and free of uncoupled Bolton-Hunter reagent (data not shown). The biological activity of the radiolabeled MDNCF was retained as assessed by the measurement of myeloperoxidase release from neutrophils (data not shown). The specific activity of labeled MDNCF was estimated to be 7×10^7 cpm/μg of protein.

Specific Binding of ^{125}I-MDNCF to Neutrophils. As shown in Fig. 1, excess amount of nonradioactive MDNCF was able to inhibit the binding of ^{125}I-MDNCF to neutrophils by >95%. This inhibitory effect of unlabeled MDNCF was dose dependent. In contrast, other chemotactic cytokines and agents such as TNF, IL-1α, FMLP, LTB4, C5a, and platelet activating factor did not exert significant inhibitory effects on binding of radiolabeled MDNCF to neutrophils, demonstrating specificity of binding of ^{125}I-MDNCF and establishing that MDNCF receptors are distinct from any other chemotactic cytokine or agent receptors.

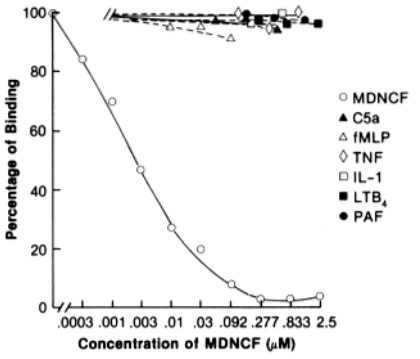

FIGURE 1. Competition for the binding of ^{125}I-MDNCF by unlabeled MDNCF, other cytokines, and chemoattractant to human neutrophils. Human neutrophils were incubated at 4°C for 1 h with 4 ng ^{125}I-MDNCF as described in Materials and Methods, in the presence of various amounts of unlabeled MDNCF, TNF, IL-1, FMLP, C5a, LT4, and platelet activating factor as indicated. The binding is expressed as a percentage of binding obtained with ^{125}I-MDNCF alone. The results represent three independent experiments.

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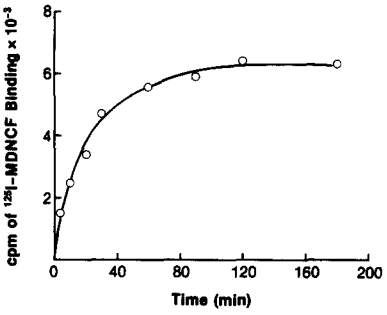

FIGURE 2. Kinetics of ^{125}I-MDNCF binding to human neutrophils. 4 ng ^{125}I-MDNCF (1.2×10^5 cpm) was incubated with human neutrophils at 4°C for indicated times. The results represent three independent experiments. Each point is the mean of duplicate determination.

Kinetics of Binding of ^{125}I-MDNCF to its Receptor. Fig. 2 shows a representative kinetic profile of the binding of ^{125}I-MDNCF to neutrophils at 4°C. The binding essentially reached equilibrium by 1 h.

Scatchard Analysis of MDNCF Receptor. The saturability of binding of ^{125}I-MDNCF to neutrophils was examined. The total binding of MDNCF increased in proportion to the amount of added labeled material. The specific binding was similarly increased (Fig. 3) and was saturated in the presence of 25 ng ^{125}I-MDNCF. The nonspecific binding of MDNCF increased linearly with increasing amounts of radiolabeled ligand. The Scatchard analysis of specific MDNCF binding at 4°C after 3 h showed a curvilinear pattern. The equilibrium K_d was 8×10^{-10} M, with the number of receptors per neutrophil estimated to be ~20,000/cell. This type of curvature and nonlinear Scatchard plot has been obtained in many receptor-ligand interactions (11). It was determined that the Hill coefficient (nH) was 1.09, indicating no significant positive or negative cooperativity in binding of MDNCF to receptors. Therefore, the MDNCF receptors seem to have single binding affinity.

Chemical Crosslinking of MDNCF with Its Receptor. Chemical crosslinking of MDNCF with its receptor was carried out using a bivalent crosslinking reagent DSS. Fig. 4 presents autoradiographs of SDS-PAGE with three different concentrations of DSS to fractionate the cell extracts under reducing conditions. Two bands of mol wt 75,000 and 67,000 were detected. These signals disappeared in the presence of excess unlabeled MDNCF, indicating that the bands represented MDNCF-specific binding molecules. Assuming that one MDNCF molecule binds to one receptor molecule, the molecular weight of MDNCF binding proteins were estimated to be 67,000 and

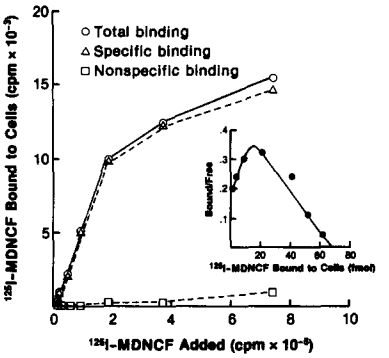

FIGURE 3. Binding isotherm of ^{125}I-MDNCF to human PMN. Neutrophils were incubated for 3 h at 4°C with various concentrations of ^{125}I-MDNCF, ranging from 0.39 to 24 nM. Nonspecific binding was determined in the presence of excess unlabeled (100-fold) MDNCF. To determine specific binding, the nonspecific binding was subtracted from the total binding. Shown in the inset is the Scatchard analysis of the binding data. The results represent three independent experiments.

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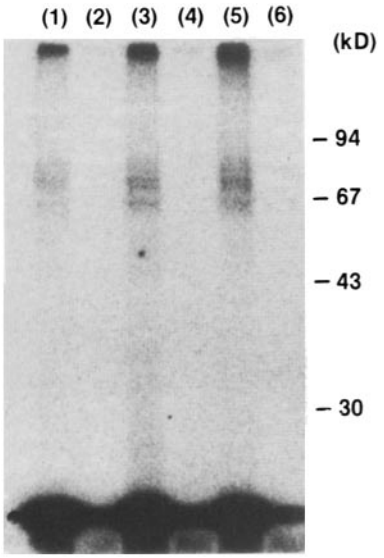

FIGURE 4. Autoradiograph of chemically crosslinked MDNCF receptor complexes from human neutrophils analyzed by SDS-PAGE (10%). Lanes *1*, *3*, and *5* present the crosslinked molecules prepared in the absence of unlabeled MDNCF using 0.75, 1, and 1.25 mg/ml DSS, respectively. Lanes *2*, *4*, and *6* present the same experiment in the presence of an 80-fold excess of unlabeled MDNCF. The molecular weight markers of the standard proteins were also run in SDS-PAGE as indicated in the figure. The results represent three independent experiments.

59,000. There was no significant difference in migration of the chemically crosslinked receptors on SDS-PAGE under reducing and nonreducing conditions (data not shown). These two bands may represent noncovalently associated MDNCF receptor subunits, proteolytically cleaved molecules, or posttranslationally modified molecules as by glycosylation, acylation, and phosphorylation.

Increase of MDNCF Receptor Expression on DMSO-treated HL60 Cells. The HL60 human promyelocytic leukemic cell line could be differentiated into myelocytes and metamyelocytes by the addition of 1.25% DMSO (8). The binding assay was first carried out using a single saturating amount (4 ng) of ^{125}I-MDNCF before and after 5 d of incubation with DMSO. In uninduced promyelocytes, the binding of the labeled material was low but was increased by fourfold in the DMSO-treated cells. Scatchard analysis performed at 4°C showed that ~7,000 receptors were present per DMSO-treated HL60 cell. The K_d was 1.2×10^{-9} M, which was similar to the K_d value for the MDNCF receptors on normal peripheral blood neutrophils (data not shown). This indicates that promyelocytes express small numbers of MDNCF receptors and that the expression of the MDNCF receptor increases with each successive stage of maturation of neutrophils, becoming maximal in the mature PMN.

In conclusion, we have reported the detection of an MDNCF-specific receptor that is distinct from the receptors for other known chemoattractants. Purification and further characterization of the MDNCF-specific receptor may provide insights into the mechanism of action of MDNCF.

Summary

Specific receptors for a recently purified and cloned monocyte-derived neutrophil chemotactic factor (MDNCF) have been identified on the surface of normal human peripheral blood neutrophils using ^{125}I-labeled recombinant human MDNCF (^{125}I-MDNCF). Competitive binding of ^{125}I-MDNCF to human neutrophils reached a maximal level at 1–3 h at 4°C. The Scatchard analysis showed that there are ~20,000

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receptors per cell with a single type of high affinity binding (K_d, 8×10^{-10} M). The receptors for MDNCF are clearly distinct from the receptors for other cytokines and chemotactic agents, e.g., IL-1α, TNF-α, and FMLP, C5a, leukotriene B4, and platelet activating factor. Based on the SDS-PAGE analysis of chemically crosslinked ^{125}I-MDNCF receptor complex, there are two polypeptides that bind MDNCF; the molecular weight of these two MDNCF receptors were estimated to be 67,000 and 59,000. Treatment of a promyelocytic cell line, HL60, with 1.25% DMSO for 5 d in vitro increased the number of receptors up to 7,000 receptors/cell with a K_d of 1.2×10^{-9} M.

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