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Successful acquisition of a neutralizing monoclonal antibody against a novel neutrophil-activating peptide, mitocryptide-1

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ABSTRACT

Mitocryptide-1 (MCT-1) is a novel neutrophil-activating peptide derived from mitochondrial cytochrome *c* oxidase subunit VIII, and its physiological role and involvement in various diseases have not yet been elucidated. Generating neutralizing antibodies against the function of MCT-1 is of particular importance for investigating its physiological and pathophysiological roles, because MCT-1 is a fragmented peptide of its mother protein and hence it is very difficult to manipulate its expression level genetically without affecting expression of the mother protein. Here, we report the successful generation of a neutralizing monoclonal antibody (MAb) against MCT-1. This MAb, designated NM1B1, which specifically bound to the region of positions 9–22 of MCT-1, showed concentration-dependent inhibition of MCT-1-induced migration and β-hexosaminidase release in neutrophilic/granulocytic differentiated HL-60 cells. Thus, NM1B1, as a neutralizing MAb against MCT-1, could elucidate not just the physiological regulatory mechanisms of MCT-1 but also its pathophysiological involvement in various inflammatory diseases *in vivo*.

1. Introduction

Many fragmented peptides are produced simultaneously in the maturation and degradation processes of functional proteins. There is accumulating evidence that considerable numbers of such fragmented peptides have a variety of biological activities that are distinct from their mother proteins. We have isolated and identified a family of novel bioactive peptides, mitocryptides, which are produced from various mitochondrial proteins and induce neutrophilic migration and phagocytosis at nanomolar concentrations [1–7]. It has also been reported that many fragmented peptides derived from various functional proteins have a variety of biological activities including stimulation of cell growth and neuronal responses [8–15]. We named these fragmented peptides with distinct biological activities from their mother proteins as “cryptides” [3]. However, little is known about their physiological and pathophysiological roles in the body, because it is difficult to investigate the functions of cryptides *in vivo* without affecting the expression levels of their mother proteins; that is, genetic manipulation of their expression inevitably influences the expression and function of their mother proteins. Therefore, it is of particular importance to overcome this difficulty. Obtaining specific neutralizing antibodies could be a promising approach to investigate the functions of cryptides *in vivo* by utilizing their specific inhibitory effects. In addition, they can also be applied to the investigation of the production and localization of cryptides in various circumstances *in vivo*.

Among neutrophil-activating cryptides, mitocryptide-1 (MCT-1) is a tricosapeptide derived from mitochondrial cytochrome *c* oxidase subunit VIII. MCT-1 was purified from porcine heart and efficiently activated neutrophils at nanomolar concentrations [1–4]. Since MCT-1 is thought to be the most abundant neutrophil-activating peptide in healthy organs [4], it is expected to be involved in the regulation of various inflammatory diseases.

In the present study, we therefore attempted to generate specific neutralizing monoclonal antibodies (MAbs) against MCT-1 to elucidate its physiological and pathophysiological significance *in vivo*.

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2. Materials and methods

2.1. Peptides

MCT-1 and its derivatives were synthesized by solid-phase peptide synthesis using the 9-fluorenylmethyloxycarbonyl method [16–19]. Synthesized peptides were proven to be more than 95% pure by analytical high-performance liquid chromatography using a COSMOSIL $5C_{18}$-AR-II column (4.6 × 150 mm; Nacalai Tesque, Inc., Kyoto, Japan). Homogeneity of the synthesized peptides was confirmed using matrix-assisted laser desorption ionization time-of-flight mass spectrometry.

2.2. Preparation of a peptide antigen

To prepare a peptide antigen, [Cys^0]MCT-1 was conjugated with bovine thyroglobulin as a carrier protein via *N*-(6-maleimidocaproyloxy)succinimide (EMCS; Dojindo Molecular Technologies, Kumamoto, Japan). Namely, the ε-amino groups of Lys residues of thyroglobulin were coupled with the *N*-hydroxysuccinimide group of EMCS in phosphate-buffered saline (PBS; pH 7.0) at 37 °C for 1 h to produce thyroglobulin-EMCS complexes. Then, the reacted solution was diluted with PBS and ultrafiltrated to remove unreacted EMCS. Thereafter, the maleimide groups of thyroglobulin-EMCS were reacted with the sulfhydryl group of [Cys^0]MCT-1 in PBS (pH 7.0) for 3 h at room temperature to obtain MCT-1-EMCS-thyroglobulin complexes. The quantity of MCT-1 bound to thyroglobulin was estimated using an Amplite™ Fluorimetric Maleimide Quantitation Kit (AAT Bioquest, CA, USA). The resultant MCT-1-EMCS-thyroglobulin complexes were used as an antigenic peptide and stored at −80 °C before use.

2.3. Production of MAbs

The animal experiments in the present study were approved by the Animal Care and Use Committee of the Nagahama Institute of Bio-Science and Technology. Eight-week-old female BALB/c mice were injected subcutaneously with the peptide antigen (per head, 7 mg MCT-1 conjugated with thyroglobulin dissolved in 300 μL PBS) mixed with 300 μL Freund's complete adjuvant (Rockland, PA, USA). The mice were boosted with the same amount of peptide antigen in PBS mixed with Freund's incomplete adjuvant (Rockland) at 7, 14, and 21 days after the first immunization. Three days prior to sacrifice, the mice were boosted intraperitoneally once with the same amount of the peptide antigen in PBS without any adjuvant.

MAbs were generated by the standard method with slight modifications [20,21]. Spleen cells obtained from immunized mice were fused with myeloma cells (PAI cells; Human Science Research Resources Bank, Tokyo, Japan) at a ratio of 10:1 using Dulbecco-PBS with 45% (w/v) polyethylene glycol 4000 (Immuno-Biological Laboratories, Gunma, Japan). Hybridoma cells were selected in GIT medium (Kohjin Bio, Saitama, Japan) containing 100 μM hypoxanthine, 0.4 μM aminopterin, and 16 μM thymidine (Sigma–Aldrich, MI, USA) for 10 days in a 96-well microtiter plate (Corning, NY, USA). Hybridoma cells producing anti-MCT-1 antibodies were screened and cloned by enzyme-linked immunosorbent assay (ELISA). ELISA plates (Thermo Fisher Scientific, MA, USA) were coated with 50 ng/well MCT-1 conjugated with keyhole-limpet hemocyanin (KLH) via EMCS (MCT-1-EMCS-KLH) in 0.1 M carbonate buffer for 16 h at 4 °C and blocked with 200 μL protein solution (5 × dilution of saturated casein solution in PBS, pH 7.2; Nacalai Tesque, Inc.) at 37 °C for 2 h. The culture supernatants (50 μL) were then added to each well of the ELISA plate and incubated for 30 min at 37 °C. After washing with PBS containing 0.05% Tween 20 (PBS-Tween) 5 times, 50 μL goat anti-mouse IgG conjugated with horseradish peroxidase (0.5 μg/mL; Life Technologies, CA, USA) were added. Then, the plates were incubated for 30 min at 37 °C, followed by washing with PBS-Tween 6 times. ABTS peroxidase substrate solution (Kirkegaard & Perry Laboratories, MD, USA) was added to each well and incubated at room temperature for 10 min in the dark to develop color. The reaction was stopped by addition of 100 μL of 1% sodium dodecyl sulfate, and absorbance was measured at 405 nm using a microplate reader (Viento XS; Bio-Tek Instruments, VT, USA). Isotypes of MAbs were confirmed by IsoStrip (Mouse Monoclonal Antibody Isotyping Kit; Roche, Basel, Switzerland) according to the manufacturer's instructions.

Each cloned hybridoma cell producing an anti-MCT-1 MAb (2.5×10^7 cells) was cultured in a flask (CELLine Flask; Corning) with GIT medium for 1 week to obtain the MAb from the culture supernatant according to the manufacture's instruction. The culture supernatant was then collected and cell debris in the supernatant was removed by centrifugation at 400 × *g* for 10 min. MAbs in the supernatant were purified by protein G affinity chromatography (Protein G Sepharose 4 Fast Flow; GE Healthcare, Little Chalfont, UK). The purified MAbs were collected and dialyzed with PBS at pH 7.4. The MAbs were dispensed into microtubes and stored at −30 °C.

2.4. Characterization of the MAbs

The binding specificity and dissociation constant (K_d) of the anti-MCT-1 MAbs were evaluated by competition ELISA [22]. Briefly, the anti-MCT-1 MAb solution was mixed with various concentrations of MCT-1, human mitocryptide-2 (hMCT-2) or *N*-formyl-L-methionyl-L-leucyl-L-phenylalanine (fMLF), and incubated at 37 °C for 2 h. The incubated solutions (50 μl) were then added to each well of the ELISA plate containing immobilized MCT-1-EMCS-KLH (150 ng/well) and incubated at 37 °C for 30 min. The anti-MCT-1 MAbs that bound to the well were evaluated subsequently by ELISA as described above. The K_d value was calculated by Scatchard plot analysis.

The antigen binding properties of anti-MCT-1 MAbs were also analyzed by competition ELISA using MCT-1 derivatives. We mixed 200 nM of the anti-MCT-1 MAbs (60 μL) with 60 μM of various MCT-1 derivatives (60 μL) and they were incubated at 37 °C for 2 h. The solutions were then added to each well of the ELISA plate with immobilized MCT-1-EMCS-KLH and further incubated for 30 min at 37 °C. The anti-MCT-1 MAbs that bound to the well were evaluated by ELISA as described above.

2.5. Evaluation of neutralizing activity

HL-60 cells (RIKEN Cell Bank, Ibaraki, Japan) were cultured in RPMI-1640 medium (Life Technologies) containing 10% fetal bovine serum (Life Technologies) in a humidified atmosphere of 5% CO_2 at 37 °C. HL-60 cells (3.2×10^7 cells/40 mL) in a 75-cm^2 flask (Asahi Glass, Chiba, Japan) were treated with 500 μM dibutyryl cyclic adenosine monophosphate (Sigma–Aldrich) for 72 h for differentiation into neutrophilic/granulocytic cells [23].

β-Hexosaminidase (β-HA) release from the differentiated HL-60 cells was analyzed as described previously [4,5] with slight modifications. In brief, differentiated HL-60 cells were washed 3 times with ice-cold HEPES-buffered Hank's solution (HBHS; 10 mM HEPES, 136.9 mM NaCl, 5.4 mM KCl, 1.2 mM $CaCl_2$, 0.44 mM KH_2PO_4, 0.49 mM $MgCl_2$, 0.41 mM $MgSO_4$, 0.34 mM Na_2HPO_4, 5.5 mM glucose, and 4.2 mM $NaHCO_3$ at pH 7.4) containing 0.1% bovine serum albumin and resuspended in HBHS at a density of 1.0×10^7 cells/mL. Then, 50 μL of the cell suspension were transferred to a tube (5.0×10^5 cells/tube) and placed on ice; DNase I

(Sigma–Aldrich) and cytochalasin B (Sigma–Aldrich) were both added to the cell suspension at a final concentration of 5 μg/mL. After pre-incubation of the cell suspension for 10 min at 37 °C, the cells were stimulated for 10 min at 37 °C with 1 μM MCT-1, 0.7 μM hMCT-2, or 3 nM fMLF solution (50 μL) pre-incubated with or without various concentrations of the anti-MCT-1 MAbs. The cells were also stimulated with MCT-1 (1 μM) pre-incubated with 1 μM of the isotype/subclass matched control MAb (LEAF™ purified mouse IgG_1, κ isotype control; BioLegend, CA, USA). Thereafter, 200 μL ice-cold reaction-quenching buffer (25 mM Tris, 123 mM NaCl, and 2.7 mM KCl at pH 7.4) were added to each cell suspension to stop stimulation. The tubes were centrifuged at 4 °C and 2300 × *g* for 1 min, and each cell supernatant was transferred to a new tube.

β-HA activity in the cell-free supernatant was determined as described previously with minor modifications [24]. Briefly, 90 μL of the supernatant obtained as described above were transferred to each well of a 96-well microtiter plate, and 60 μL of a substrate solution for β-HA (10 mM 4-nitrophenyl *N*-acetyl-β-D-glucosaminide [Sigma–Aldrich], 40 mM citrate, and 70 mM $NaHPO_4$ at pH 4.5) were added to initiate the enzyme reaction. After incubation of the plate at 37 °C for 70 min, 100 μL reaction-quenching solution (400 mM glycine at pH 10.7) were added to stop the enzyme reaction. Thereafter, the absorbance difference of each well at 415 nm for the resulting 4-nitrophenol and at 490 nm for the reference absorbance was measured. β-HA activity in each supernatant was estimated as a percentage of total enzyme activity, which was enzyme activity released after lysis of the cells with 0.05% Triton X-100. β-HA releasing activity stimulated by 60% of effective concentration (EC_{60}) of each peptide with various concentrations of the anti-MCT-1 MAbs or the isotype/subclass matched control MAb was expressed as a percentage of that without the anti-MCT-1 MAbs.

Chemotaxis of differentiated HL-60 cells was assessed as described previously [4,5] with slight modifications. In brief, differentiated HL-60 cells were washed 3 times with HBHS at 37 °C and resuspended in HBHS at a density of 4.0×10^6 cells/mL. After pre-incubation of the cell suspension at 37 °C for 10 min, 500 μL of the cell suspension were transferred to a Chemotaxicell Chamber (2.0×10^6 cells/chamber; pore size, 3 μm; Kurabo, Osaka, Japan). The chambers were placed in a 24-well microplate filled with 1 mL preheated (37 °C) HBHS containing 1 μM MCT-1, which was pre-incubated with various concentrations of the anti-MCT-1 MAbs for 2 h. The microplate was incubated for 1 h at 37 °C. The Chemotaxicell chambers were then removed from each well of the microplate, and the number of cells that had migrated into the lower side of the chambers was counted. Migration activity was estimated as a chemotaxis index; the number of cells that migrated with stimulation was divided by the number of cells that migrated without stimulation. Migration activity stimulated by 1 μM MCT-1 with various concentrations of the anti-MCT-1 MAbs was expressed as a percentage of that without the anti-MCT-1 MAbs.

2.6. Statistical analysis

Statistical comparisons were performed using Student's *t*-test (two groups). The means and the standard errors of the means (SEM) were calculated in experiments containing multiple data points. Values of $p < 0.05$ were considered statistically significant.

3. Results

3.1. Generation of anti-MCT-1 MAbs

The antibody titer against MCT-1 in the collected serum from the immunized mice increased at 1 month after the initial immunization with MCT-1-EMCS-thyroglobulin. Eleven hybridoma clones producing MAbs that showed a positive reaction against MCT-1 in ELISA were obtained by screening and cloning fused cells of splenic cells from the immunized mice and myeloma cells. One clone, MCT-1-B-1, produced a considerable amount of an MAb with higher affinity to MCT-1 than the other clones. The isotype of the purified MAb from the culture supernatant of the MCT-1-B-1 clone was identified as IgG_1. This purified MAb, designated as NM1B1, exhibited positive reactivity to the ELISA plate with immobilized MCT-1-EMCS-KLH.

3.2. Binding specificity

Since NM1B1 was shown to bind to MCT-1-EMCS-KLH, competition ELISA was performed to confirm whether it bound to MCT-1 itself. Binding of NM1B1 (30 nM) to each well of an ELISA plate with immobilized MCT-1-EMCS-KLH was reduced by free MCT-1 at concentrations above 3 μM, and was almost completely inhibited by 10 μM free MCT-1 (Fig. 1-A). These results demonstrate that MCT-1, but not linker nor KLH, actually binds to NM1B1. Competition of other neutrophil-activating peptides to the binding of NM1B1 with immobilized MCT-1-EMCS-KLH was also investigated. NM1B1 (30 nM) without any competitors bound to the well, whereas isotype/subclass-matched control MAb did not (Fig. 1-B). Free MCT-1 at a concentration of 10 μM almost completely inhibited the binding of NM1B1 to the well. On the contrary, binding of NM1B1 to the well was not prevented by 10 μM free hMCT-2 or

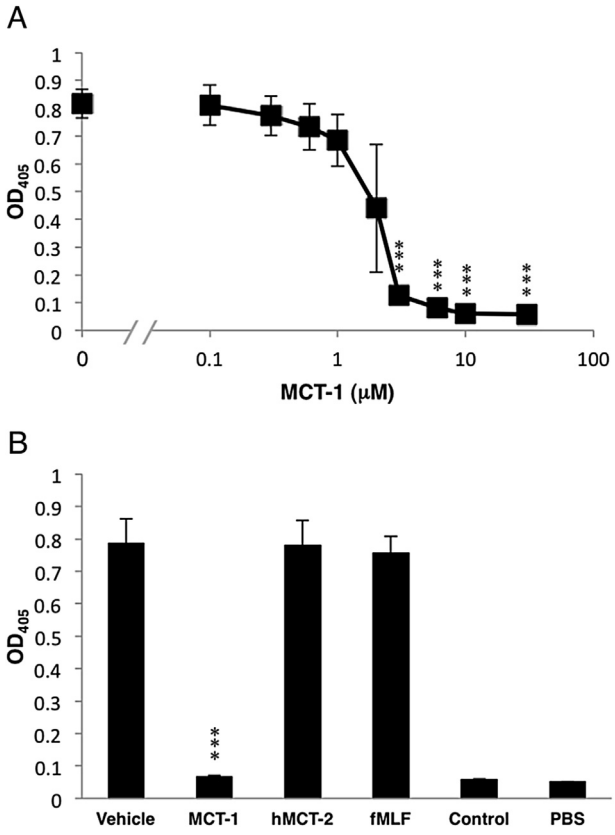

Fig. 1. Binding properties of NM1B1 to MCT-1. (A) Binding activity of NM1B1 (30 nM) to the well was examined in the presence of various concentrations of free MCT-1 as a competitor. (B) Binding specificity of NM1B1 (30 nM) was investigated by competition with MCT-1, hMCT-2, and fMLF (10 μM) to MAb binding to the well. A control MAb with the matched isotype/subclass to NM1B1 (30 nM) without competitive peptides was also tested as a negative control. ***$p < 0.001$ compared with vehicle. Data are expressed as the mean ± SEM of 3 independent experiments.

fMLF. These results indicate that NM1B1 specifically recognized MCT-1. Scatchard analysis of competitive ELISA revealed that the K_d value of antibody-peptide binding were 571 ± 54 nM.

3.3. Epitope characterization

As NM1B1 was shown to bind specifically to MCT-1, an epitope of NM1B1 was examined by competitive ELISA using an MCT-1-EMCS-KLH immobilized plate (Fig. 2). We prepared 3 fragment peptides of MCT-1: MCT-1 (1–11), MCT-1 (7–17), and MCT-1 (13–23). These peptides are 11 amino acid residues in length and overlap by 5 residues. These 3 fragment peptides spanning the entire sequence of MCT-1 were used as competitors. MCT-1 (7–17) (30 μM) inhibited binding of NM1B1 to the well, whereas MCT-1 (1–11) and MCT-1 (13–23) did not, showing that the region at positions 7–17 of MCT-1 has a part of the epitope.

To elucidate the binding site of NM1B1 more accurately, the competitive effects of MCT-1 (8–17) and MCT-1 (9–17), which were derivatives of MCT-1 (7–17) truncated at the *N*-terminal by 1 and 2 amino acids, respectively, were further examined. These two derivatives inhibited the binding of NM1B1 to the well by approximately 20%, which was almost the same extent as MCT-1 (7–17). Conversely, MCT-1 (10–17), which was a derivative truncated by 3 amino acids, did not inhibit binding at all. These results demonstrate that Trp^9 of MCT-1 is crucial for the binding of NM1B1 to the well.

Nonetheless, the inhibitory effects of MCT-1 (7–17), MCT-1 (8–17), and MCT-1 (9–17) (30 μM) were much less than that of MCT-1 itself. We also investigated the competing effects of *C*-terminal extended derivatives of MCT-1 (9–17) on binding to the well. As a result, 30 μM MCT-1 (9–18) and MCT-1 (9–19) inhibited binding to the plate by approximately 50% and 80%, respectively. These results indicate that Lys^{18} and Arg^{19} of MCT-1 are essential for NM1B1 binding. The fragments MCT-1 (9–22) and MCT-1 (9–23) inhibited the binding to the well to the similar level as MCT-1 itself. These results strongly demonstrate that the positions 9–22 region of MCT-1 is sufficient for the binding of NM1B1 to MCT-1, and at least Trp^9, Lys^{18}, and Arg^{19} of MCT-1 are essential amino acids for the epitope of NM1B1.

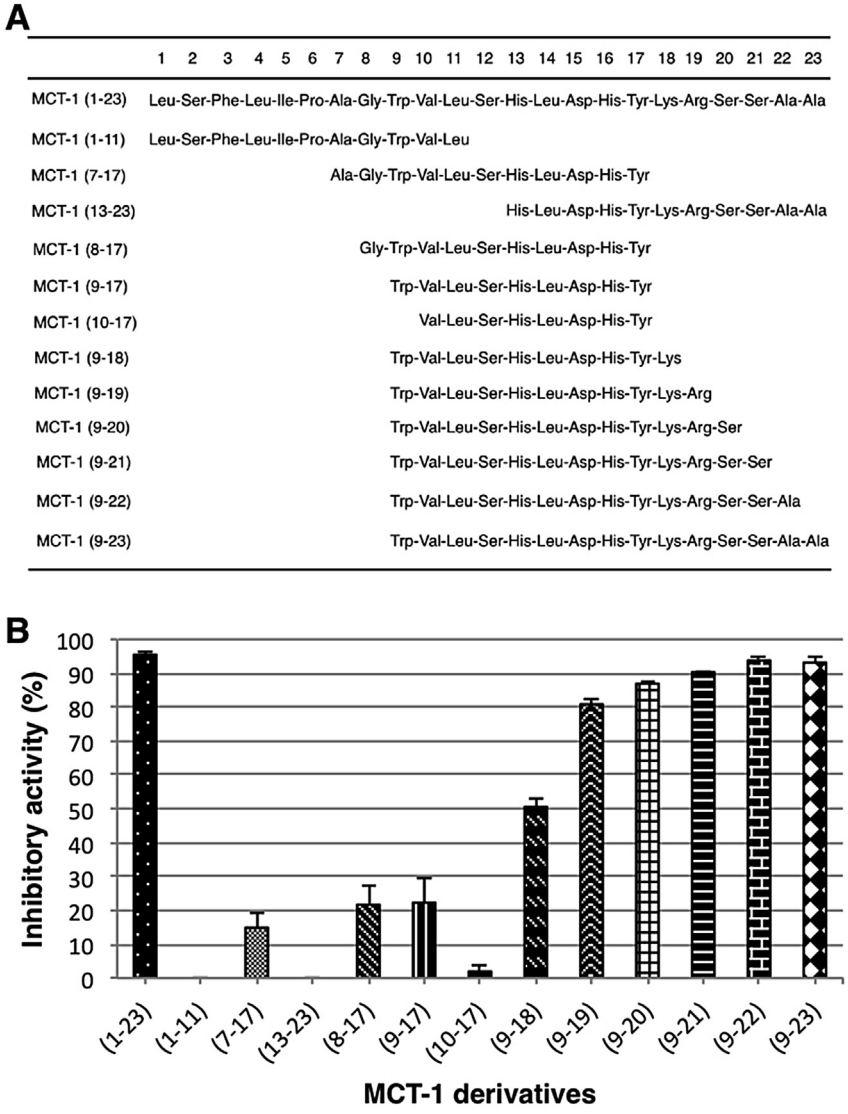

Fig. 2. Mapping the NM1B1 epitope. (A) Sequences of MCT-1 and its derivatives used for epitope mapping. (B) Inhibitory activity of MCT-1 and its derivatives (30 μM) for MAb binding to the well. Data are expressed as the mean ± SEM of 3 independent experiments.

3.4. Inhibitory effects of the anti-MCT-1 MAb on MCT-1-induced bioactivity

Since NM1B1 was shown to bind specifically to MCT-1, we investigated whether the MAb inhibited MCT-1-induced bioactivity in neutrophilic cells. The inhibitory activity of the MAb on β-HA release induced by various neutrophil-activating peptides in neutrophilic/granulocytic differentiated HL-60 cells was compared at concentrations that gave approximately 60% of the maximum effects of the peptides. As shown in Fig. 3, β-HA release induced by MCT-1 (1 μM) was reduced in a dose-dependent manner by pre-incubation of MCT-1 with NM1B1 at concentrations above 0.3 μM and was inhibited completely at 1 μM, whereas the control MAb did not block its release. NM1B1 neither altered the enzyme release caused by hMCT-2 (0.7 μM) nor fMLF (3 nM), even at a concentration as high as 1 μM. These results indicate that NM1B1 specifically inhibits β-HA release induced by MCT-1.

The inhibitory effect of NM1B1 on MCT-1-induced chemotaxis of differentiated HL-60 cells was also investigated. NM1B1 at concentrations above 0.3 μM significantly prevented cell migration (Fig. 4). These results demonstrate that NM1B1 is able to act as a specific anti-chemotaxis factor.

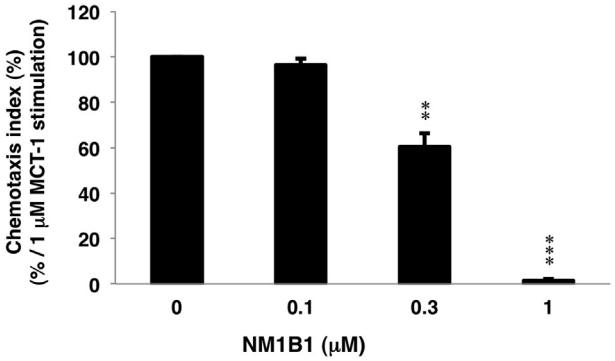

Fig. 4. Inhibitory effects of NM1B1 on chemotaxis induced by MCT-1. Neutrophilic/granulocytic differentiated HL-60 cells were stimulated using 1 μM MCT-1 with various concentrations of NM1B1 or without it. Inhibition of chemotaxis by NM1B1 was expressed as relative to the activity in the absence of the MAb. Data are expressed as the mean ± SEM of 3 independent experiments. $^{**}p < 0.01$, $^{***}p < 0.001$ compared with the chemotaxis index in the absence of the MAb.

4. Discussion

Neutrophils are involved in innate immunity by monitoring infections and tissue damage and by scavenging toxic debris at inflammatory sites [25,26]. Neutrophils immediately infiltrate damaged sites from the bloodstream to remove toxic substances, although the mechanisms underlying the transmigration and activation of neutrophils have not been elucidated well. Recently, we isolated and identified a family of neutrophil-activating cryptides, mitocryptides, including MCT-1, MCT-2, and mitocryptide-CYC, which are derived from mitochondrial proteins [1–7]. Among these mitocryptides, MCT-1 was purified and identified initially from porcine heart [4]. However, the physiological and pathophysiological roles of MCT-1 *in vivo* are still uncertain. In order to elucidate these functions of MCT-1 *in vivo*, it is valuable to investigate them by utilizing inhibition of physiological and pathophysiological phenotypes with specific neutralizing antibodies, because it is difficult to manipulate the expression level of MCT-1 genetically without affecting that of its mother protein, cytochrome *c* oxidase subunit VIII.

In the present study, we successfully obtained NM1B1 monoclonal antibody that bound specifically to MCT-1 and inhibited migration and β-HA release induced by MCT-1 in neutrophilic/granulocytic cells. These results demonstrate that NM1B1 is a specific neutralizing MAb against the biological functions of MCT-1. In addition, NM1B1 recognized amino acids at positions 9–22 of MCT-1, including the side chains of Trp^{9}, Lys^{18}, and Arg^{19}, suggesting that NM1B1 may recognize the conformational structure of the MCT-1 (9–22) region.

Interestingly, accumulating evidence shows that mitochondrial-derived contents regulate acute inflammation involving neutrophils. Mitochondrial damage-associated molecular patterns (DAMPs) that contain mitochondrial DNA and mitochondrial-derived proteinaceous molecules are released from mitochondria in the injured tissues of traumatic patients and cause systemic inflammation by promoting neutrophil activation [27,28]. Although proteinaceous molecules in mitochondrial DAMPs have not been identified, they may contain mitochondrial-derived peptides—mitocryptides, including MCT-1—because MCT-1 is considered to be the most abundant neutrophil-activating mitochondrial-derived peptide in healthy organs. Specific neutralizing MAbs against mitocryptides including NM1B1, which was obtained in the present study, are expected to be useful for the identification of the proteinaceous factors in mitochondrial DAMPs and elucidation of acute inflammation mechanisms involving mitocryptides and neutrophils. Moreover, we expect these MAbs to contribute to open a window for the functional investigation of the biological roles of cryptides that are hidden in functional protein sequences.

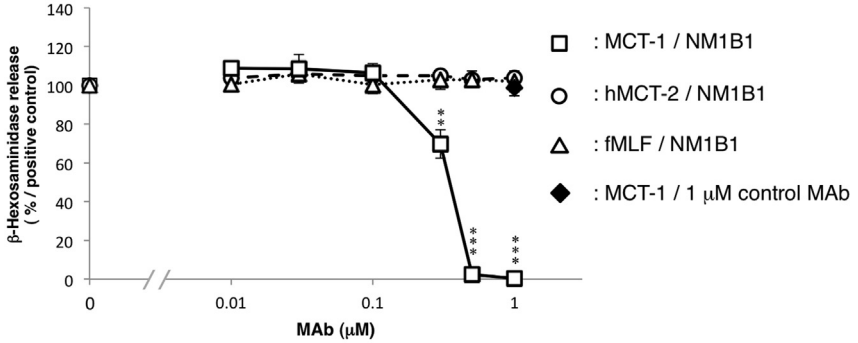

Fig. 3. Inhibitory activity of NM1B1 on the stimulation of β-HA release by MCT-1, hMCT-2, and fMLF. β-HA release from neutrophilic/granulocytic differentiated HL-60 cells stimulated by the EC_{60} of each peptide without NM1B1 was defined as the positive control. Enzyme release in the presence of various concentrations of NM1B1 or the isotype/subclass matched control MAb was expressed as a percentage of the positive control. Each value is the mean ± SEM of the duplicate determination of 6 (MCT-1 stimulation with NM1B1) or 3 (hMCT-2 or fMLF stimulation with NM1B1 and MCT-1 stimulation with control MAb) separate experiments. $^{**}p < 0.01$, $^{***}p < 0.001$ compared with each peptide stimulation without NM1B1.

In conclusion, we successfully obtained NM1B1 that binds specifically to MCT-1 and neutralizes its biological functions in neutrophilic/granulocytic cells. This neutralizing MAb is expected to be a useful tool for investigating the physiological and pathophysiological functions of MCT-1 *in vivo*.

Conflict of interest

The authors declare that there are no conflicts of interest.

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Transparency document

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