The study on physiological functions of mitocryptides, a family of neutrophil-activating peptides

一群の好中球活性化ペプチド、マイトクリプタイドの生理機能の解明に関する研究

Doctoral Thesis

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Abbreviations

Ala: L-Alanine

Asn: L-Asparagine

Arg: L-Arginine

Asp: L-Aspartic acid

Cys: L-Cysteine

Glu: L-Glutamic acid

Gly: L-Glycine

His: L-Histidine

Ile: L-Isoleucine

Leu: L-Leucine

Lys: L-Lysine

Met: L-Methionine

Phe: L-Phenylalanine

Pro: L-Proline

Ser: L-Serine

Thr: Threonine

Trp: L-Tryptophan

Tyr: L-Tyrosine

Val: L-Valine

β-HA: β-hexosaminidase

C5a: complement component 5a

EC: effective concentration

ELISA: enzyme-linked immunosorbent assay

EMCS: *N*-(6-maleimidocapryloxy)succinimide

ER: endoplasmic reticulum

fMLF: formyl-Met-Leu-Phe

FPR2: formyl peptide receptor-2

HBHS: HEPES-buffered Hank's solution

hMCT-2: human homologue of MCT-2

IL-8: interleukin 8

K_d: dissociation constant

KLH: keyhole-limpet hemocyanin

mAb: monoclonal antibody

MCT-CYC: mitocryptide-CYC

MCTs: mitocryptides

MCT-1: mitocryptide-1

MCT-2: mitocryptide-2

mtDNA: mitochondrial DNA

mtDAMPs: mitochondrial damage-associated molecular patterns

ND-6: NADH dehydrogenase subunit 6 (1-6) PBS: phosphate-buffer saline

PBS-T: PBS containing 0.05% Tween 20 pMCT-1: porcine homologue of MCT-1

pMCT-2: porcine homologue of MCT-2

RP-HPLC: reverse-phase high-performance liquid chromatography.

Chapter I

General Introduction

Endogenous bioactive peptides involve in various physiological regulations. They play physiological roles like endocrine factors such as insulin and glucagon, and paracrine factors such as somatostatin, or neurotransmitters including substance P, neurokinins, and opioid peptides. These bioactive peptides are physiologically produced and matured by specific cleavages from their inactive precursor proteins by processing enzymes in endoplasmic reticulum (ER) and trans-Golgi network, and are stored in secretory vesicles [1]. They are then released by the various physiological stimulation to regulate a variety of biological functions including neurotransmission, blood pressure, and endocrinological responses.

It is of keen interest in bioactive peptides that produced by non-ER and non-trans-Golgi network recently. Namely, functional proteins are firstly synthesized as precursor proteins on ribosomes according to mRNA transcribed from DNA. The precursor proteins are cleaved by various processing enzymes and are folded into their appropriate conformation by the assistance of molecular chaperones to exhibit their functions. After playing their physiological roles, they are inactivated by various proteases and lose their bioactivities as well as their native conformation. During these maturation and degradation process, many kinds of fragmented peptides are simultaneously produced, but physiological significance of such abundant fragmented peptides has not been of much interest for a long time [2].

Recently, it was demonstrated that some of fragmented peptides efficiently initiate innate immune responses including neutrophil activation. Namely, novel neutrophil-activating peptides, mitocryptide-1 (MCT-1), mitocryptide-2 (MCT-2), and

mitocryptide-CYC (MCT-CYC) have been isolated and identified from healthy porcine hearts (Figure I) [3–8]. MCT-1, MCT-2 and MCT-CYC were found to be endogenously fragmented peptides derived from mitochondrial cytochrome *c* oxidase subunit VIII, cytochrome *b* and cytochrome *c*, respectively. In addition, more than 50 different mitochondrial protein-derived peptides that efficiently activate neutrophilic cells have been identified utilizing bioinformatic approaches [2]. These findings propose that mitocryptides (MCTs), a novel family of neutrophil-activating peptides derived from various mitochondrial proteins, regulate innate immune responses. Such cryptic peptides with distinct bioactivities from their parent proteins have been designated as "cryptides" in general [2]. Signaling mechanisms of MCTs have been also intensively investigated in cellular levels, and it was indicated that these MCTs induce neutrophilic phagocytosis and migration via the activation of G_i-type G proteins and ERK1/2 phosphorylation [5–7]. Regarding receptor molecules for MCTs, formyl peptide receptor 2 (FPR2, also termed as formyl peptide receptor like 1) is a specific receptor for MCT-2 [7], although receptors for MCTs other than MCT-2 have not been identified yet.

After the discovery of MCTs, many fragmented peptides derived from various functional proteins including hemoglobin and proteinase-activated receptor 1, both of which efficiently induce a variety of biological functions, have been identified [9–15], and it is of particular interest to investigate their physiological significance. However, investigation of physiological production and distribution of those cryptides was very difficult, because immunostainning with antibodies that bind to their peptide sequences

can not distinguish between cryptides and their parent proteins. Furthermore, it is hard to elucidate physiological roles of those cryptides. In general, physiological involvements of endogenous bioactive peptides and functional proteins are clarified by manipulation of their genes. Since cryptides constitute a part of their parent proteins, gene manipulation of them inevitably influences the functions and expression levels of their parent proteins. To overcome these difficulties on elucidation of physiological significance of cryptides, alternative strategies are thus required to establish. If we have monoclonal antibodies (mAbs) that recognize cryptides but not their parent proteins, it would be able to analyze the production and localization of cryptides themselves. Specific neutralizing mAbs against MCTs are expected to make us investigate exactly their physiological roles *in vivo*.

In the present study, since MCT-1 is proposed to be a most abundant mitocryptide [5], I prepared specific neutralizing mAb against MCT-1. The neutralizing mAb enables us to investigate its physiological roles without the influence to the functions of its parent protein, cytochrome *c* oxidase subunit VIII (*Chapter II*). I also tried to aquire mAbs against MCT-2 because MCT-2 is an endogenous *N*-formyl peptide that is proposed to involve in innate immunity [6, 7]. Here, I obtained an mAb that recognized a cleavage site of MCT-2 from cytochrome *b*. This characteristic enables us to analyze the production and localization of MCT-2 because the mAb specifically binds to MCT-2 but not to its parent protein, cytochrome *b*. I also successfully prepared a neutralizing mAb that specifically prevents bioactivities of MCT-2. (*Chapter III*).

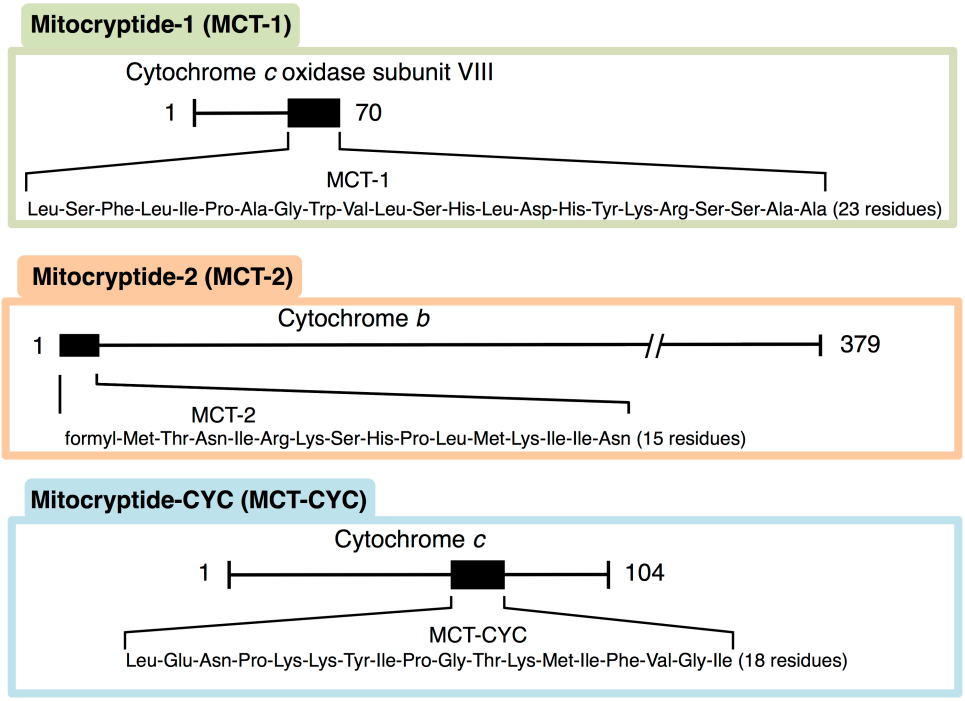

Figure I. Primary structures of MCT-1, MCT-2 and MCT-CYC derived from their parent proteins.

Chapter II

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

II-1. 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 parent protein and hence it is very difficult to manipulate its expression level genetically without affecting expression of the parent protein. Here, I describe 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*.

II-2. Introduction

Mitochondrial contents had been paid attention as pro-inflammatory factors, *i.e*. it was shown that the contents of disrupted mitochondria, which are presumably released from necrotic cells in damaged tissues, promoted neutrophilic migration [16]. However, endogenous neutrophil-activating factors had not yet been identified for a long time. To discover such neutrophil-activating substances, MCT-1 was purified and identified as endogenous neutrophil-activating peptide from healthy porcine heart [5]. MCT-1 is a tricosapeptide derived from C-terminus of mitochondrial cytochrome *c* oxidase subunit VIII (Figure I) and efficiently promotes phagocytosis and migration of neutrophils at nano-molar concentrations. However, the involvement of MCT-1 in innate immunity associating with neutrophil responses has not yet been investigated. Since MCT-1 is a peptide fragment that presumably produced by enzymatic cleavage of its parent protein, cytochrome *c* oxidase subunit VIII, silencing of this gene inevitably affects the functions and expression levels of its parent protein. In this study, I thus acquired neutralizing mAb that specifically inhibited bioactivities of MCT-1 to alternatively elucidate physiological and pathophysiological significance of MCT-1.

II-3. Materials and Methods

II-3-1. Peptides synthesis

MCT-1 and its derivatives were synthesized by a solid-phase method using a 9-fluorenylmethyloxycarbonyl strategy [17–20]. Synthesized peptides were purified by reverse-phase high-performance liquid chromatography (RP-HPLC) on a $5C_{18}$ column (20 × 250 mm; COSMOSIL, Nacalai Tesque, Inc., Kyoto, Japan). These peptides were then analyzed by RP-HPLC on a $5C_{18}$ column (4.6 × 150 mm; COSMOSIL, Nacalai Tesque, Inc.) and were demonstrated to be more than 95% pure. Molecular weights of the synthesized peptides were confirmed by matrix-assisted laser desorption ionization time-of-flight mass spectrometry.

II-3-2. 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.

II-3-3. 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 [21, 22]. 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-T) 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-T 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.

II-3-4. Characterization of the mAbs

The binding specificity and dissociation constant (K_d) of the anti-MCT-1 mAbs were evaluated by competition ELISA [23]. 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. Two hundreds nano-molar of the anti-MCT-1 mAbs (60 μL) were mixed 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.

II-3-5. 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 [24].

β-Hexosaminidase (β-HA) release from the differentiated HL-60 cells was analyzed as described previously [5, 6] 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 [25]. 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 [5, 6] 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.

II-3-6. 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.

II-4. Results

II-4-1. 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.

II-4-2. 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. II-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. II-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 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.

II-4-3. 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. II-2). I prepared 3 fragment peptides of MCT-1: MCT-1 (1–11), MCT-1 (7–17), and MCT-1 (13–23) (Fig. II-2-A). 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 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 (Fig. II-2-A). 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 (Fig. II-2-B). 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. I 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 (Fig. II-2-B). 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 (Fig. II-2-B). 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.

II-4-4. Inhibitory effects of the anti-MCT-1 mAb on MCT-1-induced bioactivity

Since NM1B1 was shown to bind specifically to MCT-1, I 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. II-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. II-4). These results demonstrate that NM1B1 is able to act as a specific anti-chemotaxis factor.

II-5. Discussion

Neutrophils are involved in innate immunity by monitoring infections and tissue damage and by scavenging toxic debris at inflammatory sites [26, 27]. Neutrophils immediately infiltrate damaged sites from the bloodstream to remove toxic substances. Some CXC chemokines including interleukin 8 (IL-8), complement-related factors such as complement component 5a (C5a), and lipid mediators such as lipoxin A_4 are known endogenous chemoattractants for neutrophils [28–30]. It has also been shown that bacterial *N*-formylated peptides and proteins induce neutrophil migration [31–33], but the mechanisms of acute inflammation involving neutrophils remain unclear. However, much evidence suggests that mitochondria-derived factors are involved in sterile immune responses. Namely, it has been demonstrated that the contents of disrupted mitochondria that are probably released from necrotic cells promote neutrophil migration [16]. Recently, a family of neutrophil-activating cryptides, mitocryptides, including MCT-1, MCT-2, and mitocryptide-CYC were isolated and identified [2–8]. Among these mitocryptides, MCT-1 was purified and identified initially from porcine heart [5]. 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 parent protein, cytochrome *c* oxidase subunit VIII.

In the present study, I successfully obtained NM1B1 mAb 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 (mtDAMPs) 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 [34, 35]. 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, I 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.

In conclusion, I 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*.

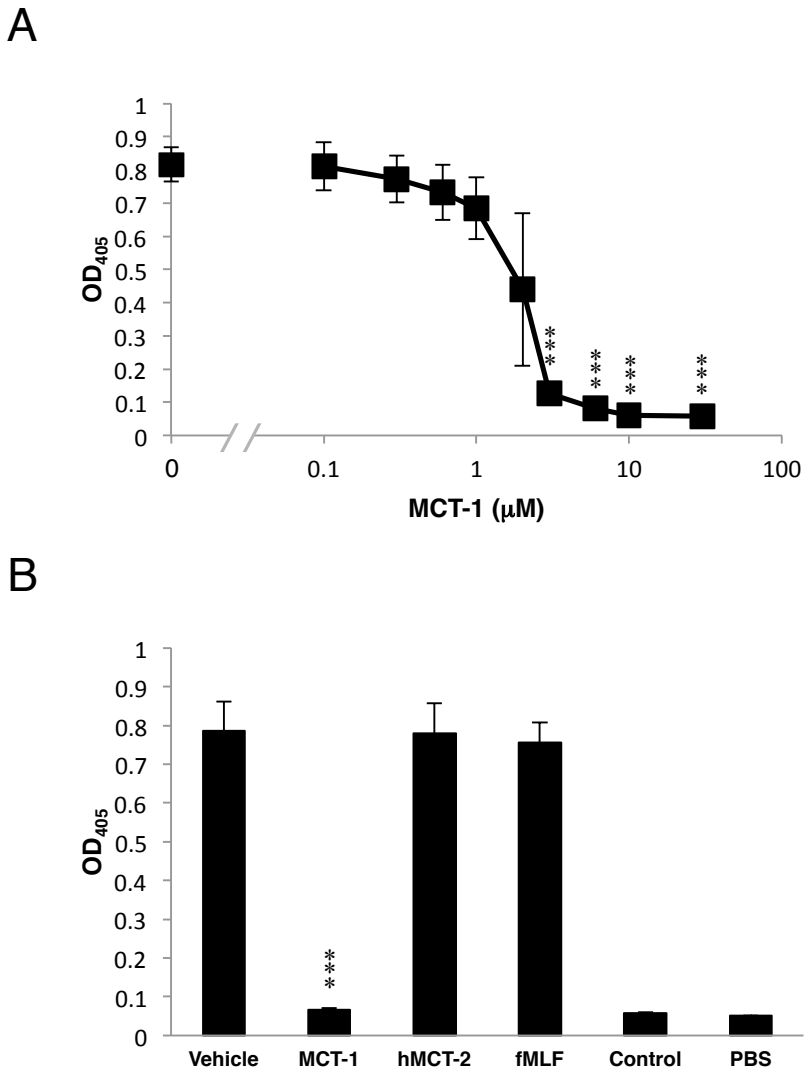

Fig. II-1. Binding properties of NM1B1 to MCT-1.

(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.

A

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23
MCT-1 (1-23)	Leu	Ser	Phe	Leu	Ile	Pro	Ala	Gly	Trp	Val	Leu	Ser	His	Leu	Asp	His	Tyr	Lys	Arg	Ser	Ser	Ala	Ala
MCT-1 (1-11)	Leu	Ser	Phe	Leu	Ile	Pro	Ala	Gly	Trp	Val	Leu												
MCT-1 (7-17)							Ala	Gly	Trp	Val	Leu	Ser	His	Leu	Asp	His	Tyr						
MCT-1 (13-23)													His	Leu	Asp	His	Tyr	Lys	Arg	Ser	Ser	Ala	Ala
MCT-1 (8-17)								Gly	Trp	Val	Leu	Ser	His	Leu	Asp	His	Tyr						
MCT-1 (9-17)									Trp	Val	Leu	Ser	His	Leu	Asp	His	Tyr						
MCT-1 (10-17)										Val	Leu	Ser	His	Leu	Asp	His	Tyr						
MCT-1 (9-18)									Trp	Val	Leu	Ser	His	Leu	Asp	His	Tyr	Lys					
MCT-1 (9-19)									Trp	Val	Leu	Ser	His	Leu	Asp	His	Tyr	Lys	Arg				
MCT-1 (9-20)									Trp	Val	Leu	Ser	His	Leu	Asp	His	Tyr	Lys	Arg	Ser			
MCT-1 (9-21)									Trp	Val	Leu	Ser	His	Leu	Asp	His	Tyr	Lys	Arg	Ser	Ser		
MCT-1 (9-22)									Trp	Val	Leu	Ser	His	Leu	Asp	His	Tyr	Lys	Arg	Ser	Ser	Ala	
MCT-1 (9-23)									Trp	Val	Leu	Ser	His	Leu	Asp	His	Tyr	Lys	Arg	Ser	Ser	Ala	Ala

B

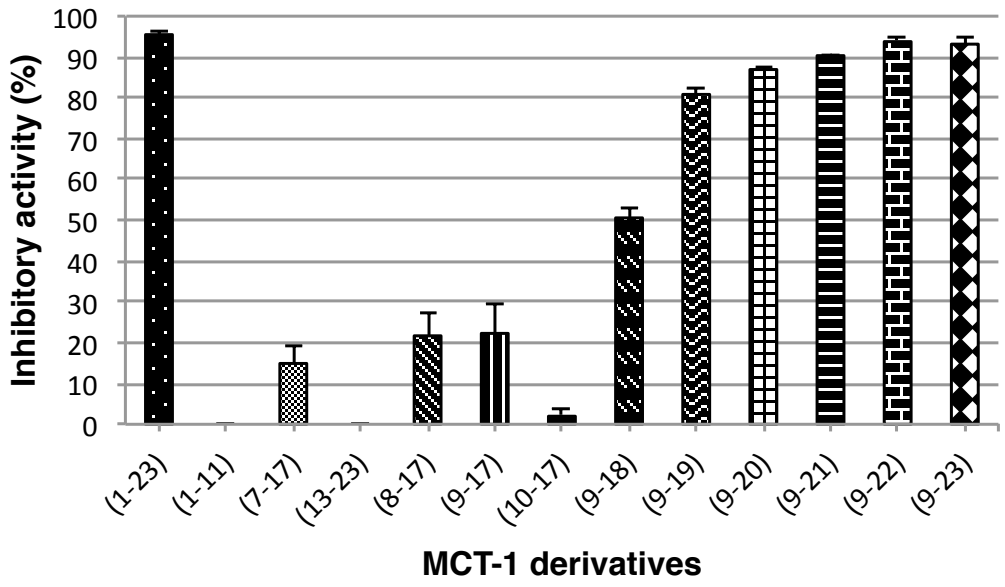

Fig. II-2. Mapping the NM1B1 epitope.

(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.

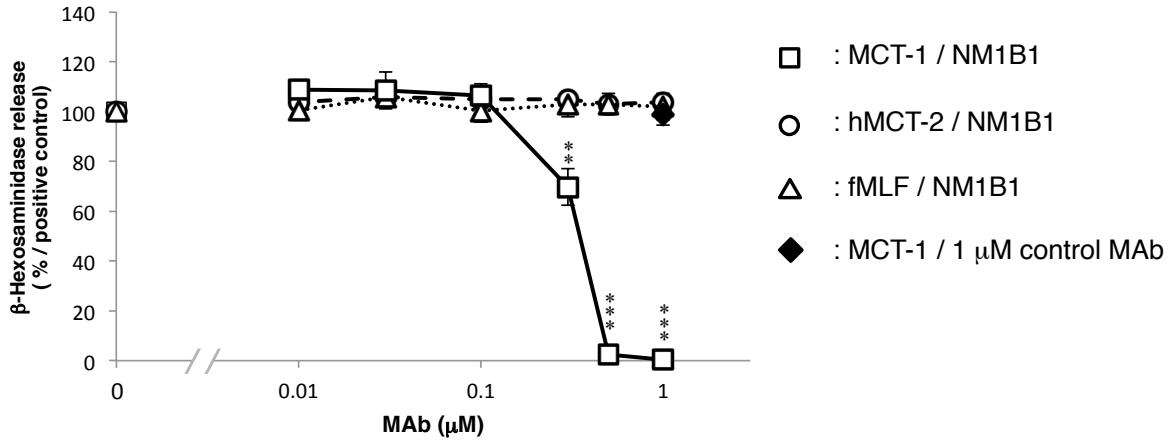

Fig. II-3. Inhibitory activity of NM1B1 on the stimulation of β-HA release by MCT-1, hMCT-2, and fMLF.

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.

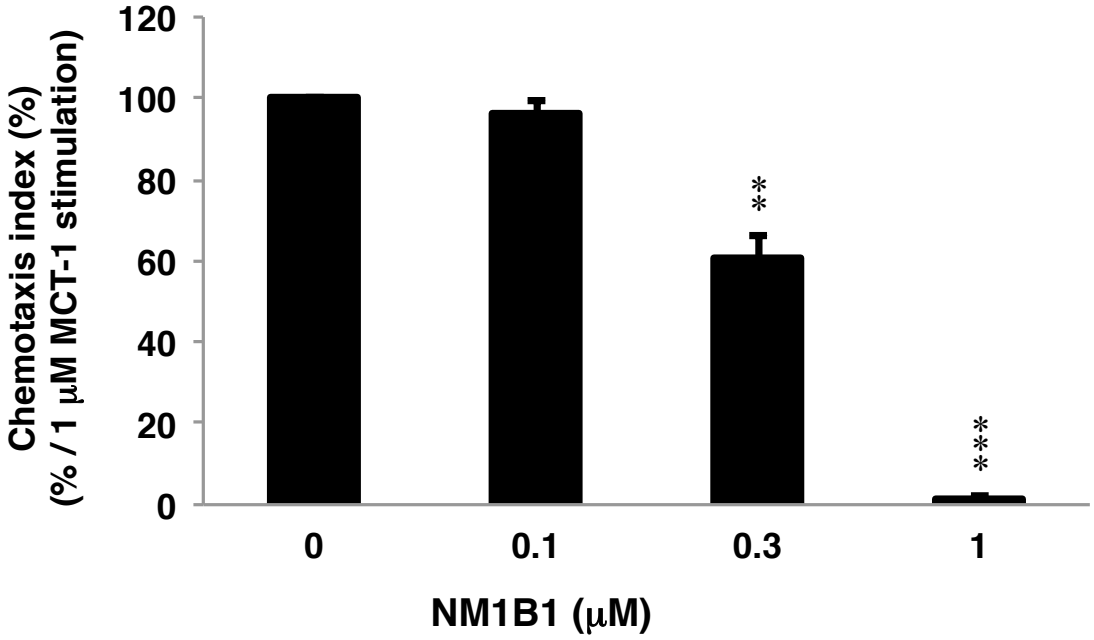

Fig. II-4. Inhibitory effects of NM1B1 on chemotaxis induced by MCT-1. Neutrophilic/granulocytic differentiated HL-60 cells were stimulated using 1 μM MCT-1 with various concentrations of NM1B1or without it. Inhibition of chemotaxis by NM1B1 was expressed as relative to the activity in the absence of 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.

Chapter III

Generation of monoclonal antibodies against mitocryptide-2: Toward a new strategy to investigate the biological roles of cryptides

III-1. Abstract

Recently a novel family of neutrophil-activating peptides including mitocryptide-1 and mitocryptide-2 (MCT-2) that endogenously produced from various mitochondrial proteins are identified. Among them, MCT-2 is an *N*-formylated pentadecapeptide derived from mitochondrial cytochrome *b* and is found to promote neutrophilic migration and phagocytosis efficiently. Signaling mechanisms of neutrophil activation by MCT-2 have been investigated at the cellular level, and MCT-2 has been demonstrated to be an endogenous specific ligand for formyl peptide receptor-2 (FPR2, also referred to as formyl peptide receptor-like 1). It was also found that MCT-2 promoted neutrophilic functions via the activation of G_{i2} proteins and phosphorylation of ERK1/2 consecutively. However, the physiological production, distribution, and functions of MCT-2 are not yet elucidated. Here, to investigate the roles of MCT-2 *in vivo*, I generated monoclonal antibodies (mAbs) against human MCT-2 (hMCT-2) that have two different characteristics. One mAb, NhM2A1, not only bound to the region of positions 10–15 of hMCT-2 but also recognized its C-terminal cleavage site that is presumably produced upon enzymatic hydrolysis of cytochrome *b*, indicating that NhM2A1 specifically interacts with hMCT-2 but not its parent protein. Moreover, I succeeded in acquiring a specific neutralizing mAb, NhM2A5, which blocks the bioactivities of hMCT-2. Specifically, NhM2A5 inhibited hMCT-2-induced β-hexosaminidase release in neutrophilic/granulocytic differentiated HL-60 cells by binding to the region of positions 5–12 of hMCT-2. Functional analysis using obtained mAbs that specifically recognize hMCT-2 but not its parent protein, cytochrome *b*, and

that neutralize bioactivities of hMCT-2 is expected to reveal the physiological roles of MCT-2, which are presently very difficult to investigate.

III-2. Introduction

Recently, mitochondrial damage-associated molecular patterns (mtDAMPs) are paid much attention as pro-inflammatory factors in sterile inflammation that were caused by internal tissue damages including ischemic injuries/infarction, burn and drug-induced liver injury [36-38]. mtDAMPs are considered to be released from the tissue injury sites, and are shown to induce neutrophil migration and activation. mtDAMPs are also proposed to be comprised of various inflammatory factors including mitochondrial DNA (mtDNA) and formyl peptides [34]. However, highly purified mtDNA was recently demonstrated to be unable to activate neutrophils *in vitro* [39, 40]. Moreover, endogenous formyl peptides in mtDAMPs have not yet been molecularly specified yet.

MCT-2 was an endogenous neutrophil-activating formylated pentadecapeptide that was isolated and identified from healthy porcine hearts [6]. It was found to be a fragmented peptide derived from N-terminus of cytochrome *b* encoded in mtDNA (Figure I) [6]. Signaling mechanisms of MCT-2 in cellular level have been intensively investigated. MCT-2 efficiently induced neutrophilic phagocytosis and migration at nano-molar concentrations via activation of G_i type G proteins associating with ERK1/2 phosphorylation [6, 7], and is also demonstrated to be a specific endogenous agonist for FPR2. Base on these findings, we propose that MCT-2 is one of activating factors in mtDAMPs. However, not only physiological production and distribution of MCT-2 but also its pathogenic involvement in sterile inflammation associating with excessive neutrophilic responses have not yet been elucidated. Here, to

establish the new strategies for investigation of physiological roles of MCT-2, I acquired mAbs with two different features. One was mAb that recognized a cleavage site in the human homolog of MCT-2 (hMCT-2, Table III-1) that is created during enzymatic hydrolysis of cytochrome *b*. This characteristic enables me to analyze the production and localization of hMCT-2 because the mAb specifically binds to hMCT-2 but not to its parent protein, cytochrome *b*. Another was mAb that specifically prevented bioactivities of hMCT-2. Fortunately, this neutralizing mAb allows me to investigate its physiological roles without influencing the functions of its parent protein.

III-3. Materials and Methods

III-3-1. Peptide synthesis

MCT-2 (porcine) and its human and mouse homologs as well as their derivative peptides were synthesized by a solid-phase method using a 9-fluorenylmethyloxycarbonyl strategy as described in *Chapter II* [17–20]. Purified 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.

III-3-2. Preparation of peptide antigen

The hMCT-2 antigen was prepared by conjugation with an hMCT-2 derivative, which was modified by the addition of a Cys residue at the N-terminus ([Cys^0]hMCT-2), and thyroglobulin as carrier protein via *N*-(6-maleimidocaproyloxy)succinimide (EMCS, Dojindo Molecular Technologies, Kumamoto, Japan), as described previously [41]. Briefly, bovine thyroglobulin (Sigma-Aldrich, MO, USA) was reacted with EMCS in phosphate-buffered saline (PBS, pH 7.0) at 37°C for 1 h to prepare thyroglobulin-EMCS complexes. The sulfhydryl group of [Cys^0]hMCT-2 was then coupled with the maleimide group of thyroglobulin-EMCS in PBS (pH 7.0) at room temperature for 3 h. The obtained peptide antigen, hMCT-2-EMCS-thyroglobulin, was store at –80°C until use.

III-3-3. Development of mAbs

The animal experiments were conducted under the guidance of Animal Care and Use Committee of the Nagahama Institute of Bio-Science and Technology (Approval No. 047). An emulsion of the peptide antigen was prepared by mixing with the peptide antigen described above and Freund's complete adjuvant (Rockland, PA, USA). Eight-week-old female BALB/c mice (Oriental Bio Service, Kyoto, Japan) were subcutaneously injected with the emulsion. After the first immunization, these mice were boosted with an emulsion composed of an identical amount of the peptide antigen and Freund's incomplete adjuvant (Rockland) once weekly for 1 month. For the final immunization, these mice were intraperitoneally injected with the peptide antigen 3 days before sacrifice.

mAbs against hMCT-2 were generated according to standard methods with minor modifications [21, 22, 41]. Spleen cells from immunized mice and PAI cells (Human Science Research Resource Bank, Tokyo, Japan) were mixed at a ratio of 10:1. Then, cell fusion was performed with 45% (w/v) polyethylene glycol 4000 (Immuno-Biological Laboratories, Gunma, Japan) in Dulbecco's PBS. The produced 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) for 10 days at 37°C and 5% CO_2 in a humidified atmosphere. Thereafter, hybridoma clones producing antibodies against hMCT-2 were screened by enzyme-linked immunosorbent assay (ELISA) using 96-well plates with immobilized hMCT-2 antigen as described below and cloned by limiting dilution. Isotypes of anti-hMCT-2 mAbs were examined

by IsoStrip (Mouse Monoclonal Antibody Isotyping Kit, Roche, Basel, Switzerland) according to the manufacturer's instructions.

The cloned hybridoma cells producing anti-hMCT-2 mAbs were cultured in a CELLine Flask (Corning, NY, USA) using GIT medium for 1 week. The mAb in the cell culture supernatant was purified using Protein G Sepharose 4 Fast Flow (GE Healthcare, Little Chalfont, UK). The purified mAb was dialyzed in PBS (pH 7.4) and stored at –30°C before use.

III-3-4. ELISA

Ninety-six-well microtiter plates were immobilized with hMCT-2 conjugated with keyhole-limpet hemocyanin (KLH) via EMCS (hMCT-2-EMCS-KLH) at a concentration of 50 ng/well in 0.1 M carbonate buffer (0.1 M Na_2CO_3 / $NaHCO_3$, pH 9.5) at 4°C for 18 h. Plates were then blocked with protein solution (200 μl of 5-fold dilution of saturated casein solution in PBS [Nacalai Tesque, Inc.]) for 2 h at 37°C, and washed with PBS containing 0.05% Tween 20 (PBS-T) 4 times. Anti-hMCT-2 mAb solution (50 μl of cell culture supernatant or purified mAb solution) was added, and the plates were incubated at 37°C for 30 min. After washing the plates 5 times with PBS-T, 50 μl of goat anti-mouse IgG conjugated with horseradish peroxidase (0.5 μg/ml, Life Technologies, CA, USA) was added and the plates were incubated for 30 min at 37°C. The plates were again washed with PBS-T 6 times, and developed using 100 μl/well of ABTS peroxidase substrate solution (Kirkegaard & Perry Laboratories, Inc., MD, USA) at room temperature in the dark for 10 min. The enzyme reaction was stopped by

addition of 100 μl/well of 1% sodium dodecyl sulfate and absorbance at 405 nm was measured in a microtiter plate reader (Viento XS, BioTek Instruments, VT, USA).

III-3-5. Binding specificity

Binding specificity of the anti-hMCT-2 mAbs was evaluated by competition ELISA. Anti-hMCT-2 mAbs were mixed with various competitor peptides. The mixture was incubated at 37°C for 2 h and then added to microtiter plates with immobilized hMCT-2-EMCS-KLH. Anti-hMCT-2 mAbs that bound to the plates were evaluated by ELISA as described above. The dissociation constant (K_d) of the anti-hMCT-2 mAbs was calculated by Scatchard analysis.

III-3-6. Epitope mapping

Epitopes of hMCT-2 for the binding to mAbs were also examined by competition ELISA using hMCT-2 derivatives with single-residue Ala substitutions and fragment derivatives of hMCT-2. Anti-hMCT-2 mAb was incubated with hMCT-2 or each hMCT-2 derivative at 37°C for 2 h. The solution was added to each well of the plate with immobilized hMCT-2-EMCS-KLH, and the plate was further incubated at 37°C for 30 min. The mAb binding to the plate was examined by ELISA as described above.

III-3-7. Inhibitory potency of mAb on the activation of neutrophilic cells

HL-60 cells (RIKEN Cell Bank, Ibaraki, Japan) were cultured in RPMI-1640

medium (Thermo Fisher Scientific, MA, USA) containing 10% fetal bovine serum (Thermo Fisher Scientific) in a humidified atmosphere at 37°C and 5% CO_2. The cells were treated with 500 μM dibutyryl cyclic adenosine monophosphate (Sigma-Aldrich) for 72 h to differentiate the cells into neutrophilic/granulocytic cells as described elsewhere [5, 24].

The inhibitory effects of anti-hMCT-2 mAb on hMCT-2-induced β-hexosaminidase (β-HA) release were evaluated in HL-60 cells differentiated into neutrophilic/granulocytic cells [41]. 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 $NaHCO_3$, pH 7.4) containing 0.1% bovine serum albumin. Then, the cells were resuspended in HBHS at a density of 1.0×10^7 cells/ml and DNase I (Sigma-Aldrich) and cytochalasin B (Sigma-Aldrich) were each added at a final concentration of 5 μg/ml. The cell suspension (5.0×10^5 cells/50 μl/tube) was pre-incubated at 37°C for 10 min. Then, the cells were stimulated with 50 μl of peptide solution that had been pre-incubated with or without various concentrations of anti-hMCT-2 mAb at 37°C for 2 h. The stimulation was performed at 37°C for 10 min. These cells were also stimulated at 37°C for 10 min with 50 μl of hMCT-2 solution that had been pre-incubated with control mAb (LEAF purified mouse IgG_1, κ isotype control; BioLegend, CA, USA) at 37°C for 2 h. The stimulation was terminated by adding 200 μl of ice-cold reaction quenching buffer (25 mM Tris, 123 mM NaCl, and 2.7 mM KCl, pH 7.4) to each cell suspension. These tubes were

centrifuged at 4°C and 2,300 × *g* for 1 min, and each cell-free supernatant was transferred into a new tube.

β-HA activity in the cell-free supernatant was evaluated as described previously [5, 41]. Briefly, 90 μl of the cell-free supernatant was transferred to each well of a 96-well microtiter plate, and 60 μl of a substrate solution (10 mM 4-nitrophenyl *N*-acetyl-β-D-glucosaminide [Sigma-Aldrich] in 40 mM citrate and 70 mM $NaHPO_4$, pH 4.5) was added for initiation of the enzyme reaction. After incubation of the plate at 37°C for 60 min, 100 μl of 400 mM glycine (pH 10.7) was added to stop the enzyme reaction. The absorbance at 415 nm and 490 nm (for reference) was measured by microtiter plate reader (Viento XS, BioTek Instruments). The β-HA release induced by each peptide in the presence of various concentrations of anti-hMCT-2 mAb or control mAb was estimated as a percentage of the stimulation of each peptide without mAbs.

III-3-8. Statistical analysis

The data in the present study are expressed as means ± standard error (SE) in experiments containing multiple data points. Statistical comparisons between two groups were performed using Student's *t*-test, and $P < 0.05$ was considered significant.

III-4. Results

III-4-1. Generation of anti-hMCT-2 mAbs

It is important to obtain peptide-specific antibodies against hMCT-2 that recognize the cleavage site of hMCT-2 that is presumably generated upon enzymatic hydrolysis of its parent protein, cytochrome *b*, for investigation of its physiological production and distribution. Moreover, it is crucial to acquire neutralizing antibodies against hMCT-2 for elucidation of its physiological and pathophysiological roles because hMCT-2 is a bioactive peptide hidden in its parent protein. To prepare those mAbs, hybridoma cells were generated by cell fusion, utilizing spleen cells of mice immunized with hMCT-2-EMCS-thyroglobulin, and cloned by limiting dilution. I obtained several clones producing mAbs that bound to hMCT-2. I then screened features of these mAbs regarding their recognition of the cleavage site and the neutralizing potency against the biological functions of hMCT-2. As a result, I acquired one clone producing mAb (NhM2A1) that recognized the cleavage site and another clone that produced the neutralizing mAb (NhM2A5). I found that the binding of NhM2A1 to the plate coated with hMCT-2-EMCS-KLH was inhibited by free hMCT-2, but not an hMCT-2 derivative that was elongated by one amino acid at its C-terminus (data not shown), suggesting that NhM2A1 recognized the C-terminal cleavage site on hMCT-2. I also screened inhibitory potencies of anti-hMCT-2 mAbs in cell culture medium on hMCT-2-induced β-HA release in HL-60 cells differentiated into neutrophilic/granulocytic cells. NhM2A5 produced from one of the clones prevented hMCT-2-induced β-HA release (data not shown). NhM2A1 and NhM2A5 were then

purified from the culture medium of those hybridoma clones, and I confirmed that both isotypes were IgG_1 κ by Isostrip.

III-4-2. Characterization of anti-hMCT-2 mAb recognizing a cleavage site on hMCT-2

Binding of NhM2A1 (3 nM) to the plate with immobilized hMCT-2-EMCS-KLH was significantly prevented by free hMCT-2 (10 μM) (Fig. III-1-A). In contrast, other neutrophil-activating peptides, porcine MCT-1 (pMCT-1), NADH dehydrogenase subunit 6 (1-6) (ND-6), and formyl-Met-Leu-Phe (fMLF) at 10 μM did not inhibit the mAb binding (Table III-1, Fig. III-1-A). Cross-binding of NhM2A1 to MCT-2 homologs was also examined by competition ELISA. NhM2A1 binding to the plate was prevented by hMCT-2 in concentration-dependent manner (Fig. III-1-B). Porcine and mouse MCT-2 homologs (Table III-1) also dose-dependently inhibited the binding, but inhibitory effects were lower than that of hMCT-2 (Fig. III-1-B). These results suggest that NhM2A1 binds to hMCT-2 and also can recognize porcine MCT-2 and mouse MCT-2. The K_d value of NhM2A1 on the binding to hMCT-2 estimated by Scatchard analysis on competition ELISA was 8.2 ± 1.7 nM.

Epitope mapping of NhM2A1 was performed by competition ELISA using fragment derivatives of hMCT-2. The hMCT-2 (9–15) peptide dose-dependently prevented NhM2A1 binding (10 nM) to the plate with immobilized hMCT-2-EMCS-KLH, but hMCT-2 (1–7) and hMCT-2 (5–11) did not (Fig. III-2-A). These results indicate that the epitope is located in the C-terminal region of hMCT-2.

hMCT-2 (10–15), hMCT-2 (11–15), and hMCT-2 (12–15), which are truncated derivatives of hMCT-2 (9–15), were then examined for the elucidation of its epitope. The hMCT-2 (9–15) and hMCT-2 (10–15) peptides inhibited NhM2A1 binding to the plate with similar potencies. On the other hand, the inhibitory effects of hMCT-2 (11–15) and hMCT-2 (12–15) were dramatically decreased in comparison with that of hMCT-2 (10–15). These results demonstrate that an NhM2A1-binding epitope is located within hMCT-2 (10–15).

Since NhM2A1 bound to hMCT-2 (10–15), it was also investigated whether NhM2A1 recognized a cleavage site in hMCT-2 that is generated upon its hydrolysis from cytochrome *b* (Fig. III-2-B). NhM2A1 binding (10 nM) to the plate was almost completely prevented by 3 μM hMCT-2 (1–15). In contrast, 3 μM hMCT-2 (1–16), hMCT-2 (1–17), and hMCT-2 (1–20), which are hMCT-2 derivatives that are C-terminally elongated with the same amino acid residues that cytochrome *b* has, did not inhibit the binding of NhM2A1 to the plate (Fig. III-2-B). These results indicate that NhM2A1 recognizes this C-terminal cleavage site on hMCT-2.

III-4-3. Characterization of neutralizing mAb against hMCT-2

Binding of NhM2A5 (10 nM) to the plate with immobilized hMCT-2-EMCS-KLH was significantly inhibited by free hMCT-2 (10 μM), but not by other neutrophil-activating peptides, pMCT-1, ND-6, and fMLF (Fig. III-3-A). Cross-binding of NhM2A5 to MCT-2 homologs was also investigated by competition ELISA, and it was shown that porcine MCT-2, mouse MCT-2, and control peptide

(pMCT-1) did not inhibit the mAb binding (Fig. III-3-B). These results demonstrate that NhM2A5 is a specific mAb against hMCT-2. The K_d value of NhM2A5 on the binding to hMCT-2 calculated by Scatchard analysis on competition ELISA was 3.1 ± 3.5 nM.

Epitopes recognized by NhM2A5 were investigated by competition ELISA using hMCT-2 derivatives with single-residue Ala substitutions. Inhibitory activities of the derivatives with Ala at position 5, 6, 7, 9, 10, 11, or 12 were significantly decreased (Fig. III-4-A), whereas the substitutions at the other positions did not affect the inhibitory potency. These results indicate that the side chains at positions 5, 6, 7, 9, 10, 11 and 12 of hMCT-2 are involved in NhM2A5 binding to hMCT-2.

Moreover, an epitope involved in the interaction between NhM2A1 and hMCT-2 was confirmed by competition ELISA using hMCT-2 (5–12). hMCT-2 (5–12) as well as hMCT-2 (1–15) dose-dependently prevented NhM2A5 binding (10 nM) to the plate coated with hMCT-2-EMCS-KLH with the same potency (Fig. III-4-B). These results demonstrate that the required structure of hMCT-2 for NhM2A1 binding is located in the region from positions 5 to 12 of hMCT-2 and the side chains of amino acids at positions 5, 6, 7, 9, 10, 11, and 12 are involved.

III-4-4. Inhibitory potency

Because NhM2A5 in the culture medium of a hybridoma clone qualitatively prevented the bioactivity of hMCT-2 as described above, I investigated whether the mAb inhibited hMCT-2-induced β-HA release from HL-60 cells differentiated into neutrophilic/granulocytic cells. As shown in Fig. III-5, β-HA release induced by

hMCT-2 (20 nM) was reduced by NhM2A5 in a concentration-dependent manner, whereas it was unaffected by control mAb. NhM2A5 did not influence β-HA release promoted by pMCT-1 (200 nM), ND-6 (2 nM), or fMLF (2 nM), even at 300 nM. These results demonstrate that NhM2A5 specifically inhibits β-HA release induced by hMCT-2, indicating that NhM2A5 is a specific neutralizing mAb against the biological functions of hMCT-2.

III-5. Discussion

In the present study, I succeeded in obtaining a peptide-specific anti-hMCT-2 mAb (NhM2A1), which recognizes a cleavage site of hMCT-2 that is generated upon enzymatic hydrolysis of its parent protein, cytochrome *b*. NhM2A1 was also able to bind not only to hMCT-2 but also to porcine and mouse MCT-2, although the binding affinities of the porcine and mouse homologs were lower than that of hMCT-2 (Fig. III-1-B). However, the affinities of NhM2A1 for the porcine and mouse MCT-2 homologs were considered sufficient for their detection, because the K_d value of NhM2A1 against hMCT-2 was 8.2 ± 1.7 nM and the inhibitory effects of porcine and mouse MCT-2 on NhM2A1 binding to the plate with immobilized hMCT-2-EMCS-KLH were from one-third to one-tenth that of hMCT-2 (Fig. III-1-B). These findings demonstrate that NhM2A1 can detect hMCT-2 and its mammalian homologs without recognizing its parent protein. NhM2A1 is thus expected to be useful for immunostaining of MCT-2; that is, NhM2A1 enables examination of the production and distribution of MCT-2 without detecting its parent protein, not only at the cellular level but also in various tissues.

Here, I also succeeded in obtaining a specific neutralizing mAb (NhM2A5) against the functions of hMCT-2 but not those of other neutrophil-activating peptides (Fig. III-5). The epitope of NhM2A5 was found to be in positions 5 to 12 of hMCT-2, indicating that the mAb bound to the central region of hMCT-2 (Fig. III-4). Prior investigation of the structure-activity relationships of hMCT-2 in its activation of neutrophilic cells revealed that hMCT-2 (1–5) was the minimum structure needed to

induce β-HA release [42]. The study also showed that the decapeptide sequence from positions 6 to 15 of hMCT-2 contribute to the binding affinity between hMCT-2 and its receptor, FPR2. Taken together, these findings suggest that NhM2A5 inhibits the bioactivities of hMCT-2 by binding to not only the minimum structure containing the activation site for FPR2 but also the affinity-involving region of hMCT-2. I successfully acquired NhM2A1 that binds to the cleavage site of hMCT-2 and NhM2A5 that neutralizes the bioactivity of hMCT-2; thus, it has become possible to investigate the physiological functions, production, and distribution of hMCT-2. Recently, mitochondrial damage-associated molecular patterns (mtDAMPs) have been focused on as inflammation-inducing factors [34–36, 43, 44]. mtDAMPs are proposed to be composed of formyl peptides and mtDNA and induce sterile inflammation associated with neutrophil migration and activation. However, it has recently been demonstrated that highly purified mtDNA does not promote neutrophil activation [39, 40]. On the other hand, it has been shown that not only MCT-1 but also MCT-2 and other *N*-formylated peptides derived from mitochondrial proteins encoded in mitochondrial DNA (mtDNA) promote activation of neutrophilic cells [5, 6, 45–47]. However, the molecules responsible for inducing innate immunological responses including neutrophil functions have not been identified *in vivo*. Recently, using the NhM2A1 mAb I found hMCT-2 present in mtDAMPs (manuscript in preparation), and are now investigating the contribution of hMCT-2 to mtDAMP-induced innate immunity by utilizing the inhibitory effects of NhM2A5. These approaches will contribute to elucidating the bioactive factors in mtDAMPs.

In conclusion, I sought to establish strategies that enable investigation of the physiological roles of hMCT-2 by generating mAbs that recognize its cleavage site and that neutralize its physiological functions. The approaches utilizing these mAbs are expected to open a new era for the investigation of physiological significance of not only MCTs but also cryptides.

Table III-1. Amino acid sequences of MCTs and other neutrophil-activating peptides

MCT-1 (Porcine):	H-Leu-Ser-Phe-Leu-Ile-Pro-Ala-Gly-Trp-Val-Leu-Ser-His-Leu-Asp-His-Tyr-Lys-Arg-Ser-Ser-Ala-Ala-OH
MCT-2 (Porcine):	formyl-Met-Thr-Asn-Ile-Arg-Lys-Ser-His-Pro-Leu-Met-Lys-Ile-Ile-Asn-OH
MCT-2 (Human):	formyl-Met-Thr-Pro-Met-Arg-Lys-Ile-Asn-Pro-Leu-Met-Lys-Leu-Ile-Asn-OH
MCT-2 (Mouse):	formyl-Met-Thr-Asn-Met-Arg-Lys-Thr-His-Pro-Leu-Phe-Lys-Ile-Ile-Asn-OH
ND-6 (1-6):	formyl-Met-Met-Tyr-Ala-Leu-Phe-OH
fMLF:	formyl-Met-Leu-Phe-OH

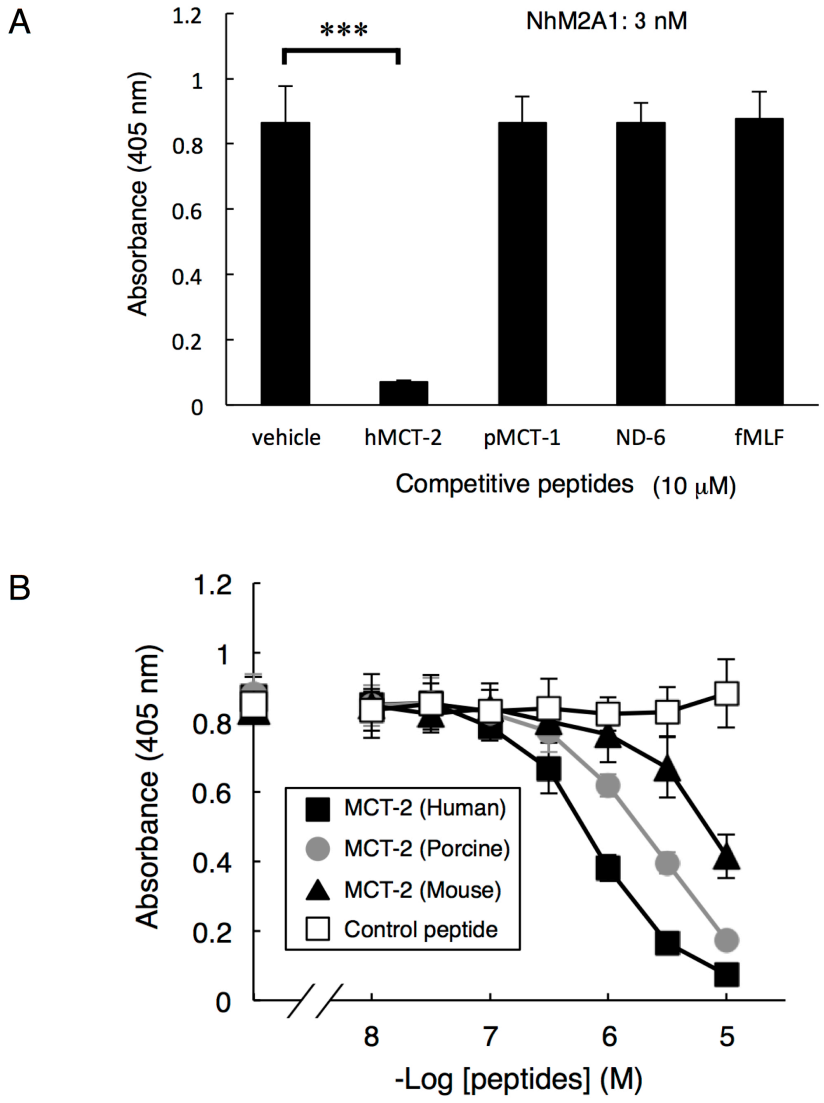

Fig. III-1. Binding specificity of NhM2A1. (A) NhM2A1 binding (3 nM) to a 96-well plate with immobilized hMCT-2-EMCS-KLH was investigated by competition ELISA using hMCT-2, pMCT-1, ND-6, and fMLF as competitive peptides (10 μM). (B) NhM2A1 binding (3 nM) to the plate was examined by competition ELISA using human MCT-2, porcine MCT-2, mouse MCT-2, and porcine MCT-1 (control peptide) as competitive peptides (10 μM). ***$P < 0.001$ compared with vehicle. Data are expressed as means ± SE of 3 independent experiments.

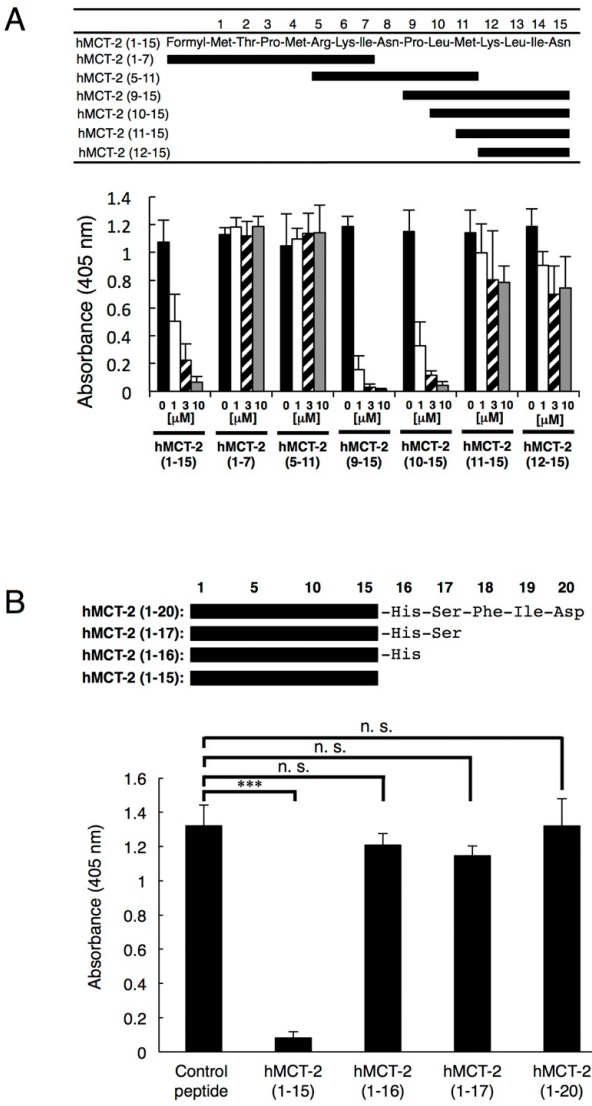

Fig. III-2. Mapping of the NhM2A1 epitope. (A) NhM2A1 epitope was investigated by competition ELISA using hMCT-2 and its derivatives on binding of NhM2A1 (10 nM) to a 96-well plate with immobilized hMCT-2-EMCS-KLH. (B) Recognition by NhM2A1 of the C-terminal cleavage site of hMCT-2 was examined by competition ELISA using hMCT-2 derivatives that were elongated with amino acid residues based on the sequence of the parent polypeptide, cytochrome *b*, at its C-terminus. ***$P <$ 0.001 compared with control peptide (porcine MCT-1). Data are expressed as means ± SE of 3 separate experiments. n.s., not significant.

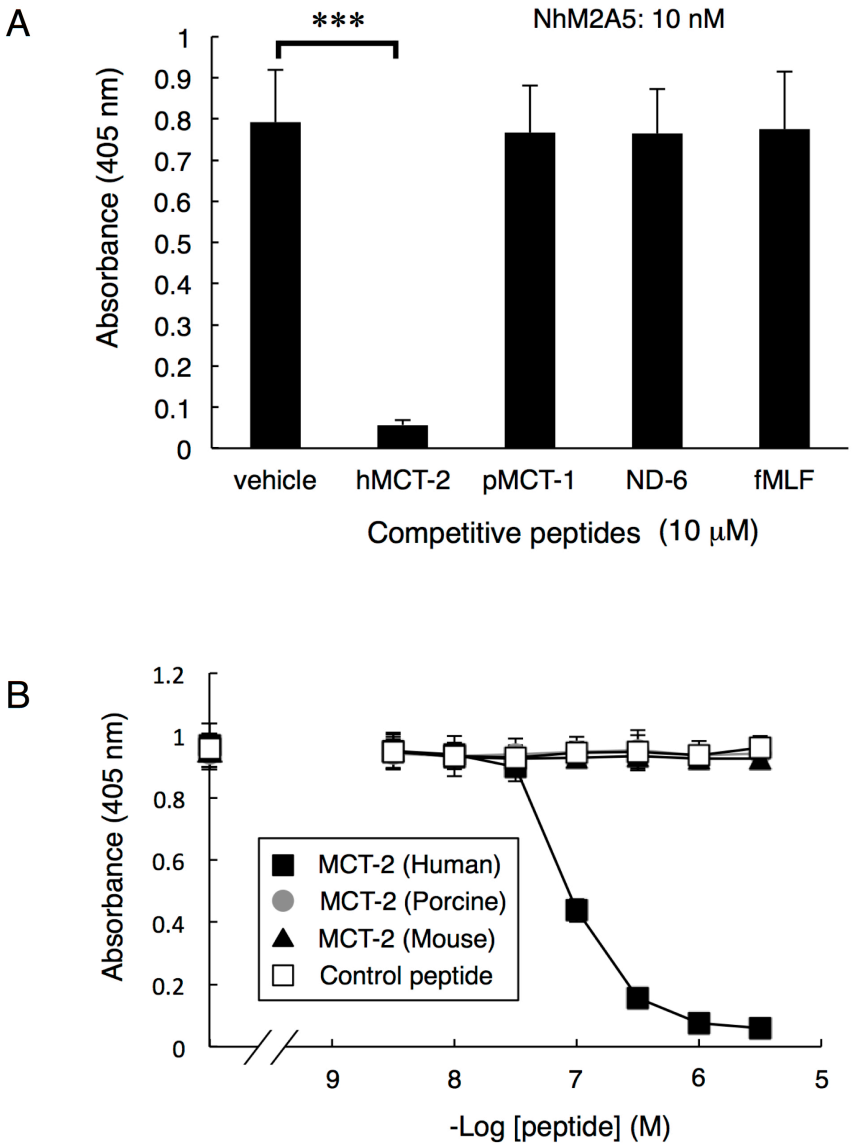

Fig. III-3. Binding specificity of NhM2A5. (A) NhM2A5 binding (10 nM) to a 96-well plate with immobilized hMCT-2-EMCS-KLH was examined by competition ELISA utilizing hMCT-2, pMCT-1, ND-6, and fMLF as competitive peptides (10 μM). (B) NhM2A5 binding (10 nM) to the plate was investigated by competition ELISA using human MCT-2, porcine MCT-2, mouse MCT-2, and porcine MCT-1 (control peptide) as competitive peptides (10 μM). ***P < 0.001 compared with vehicle. Data are expressed as means ± SE of 3 independent experiments.

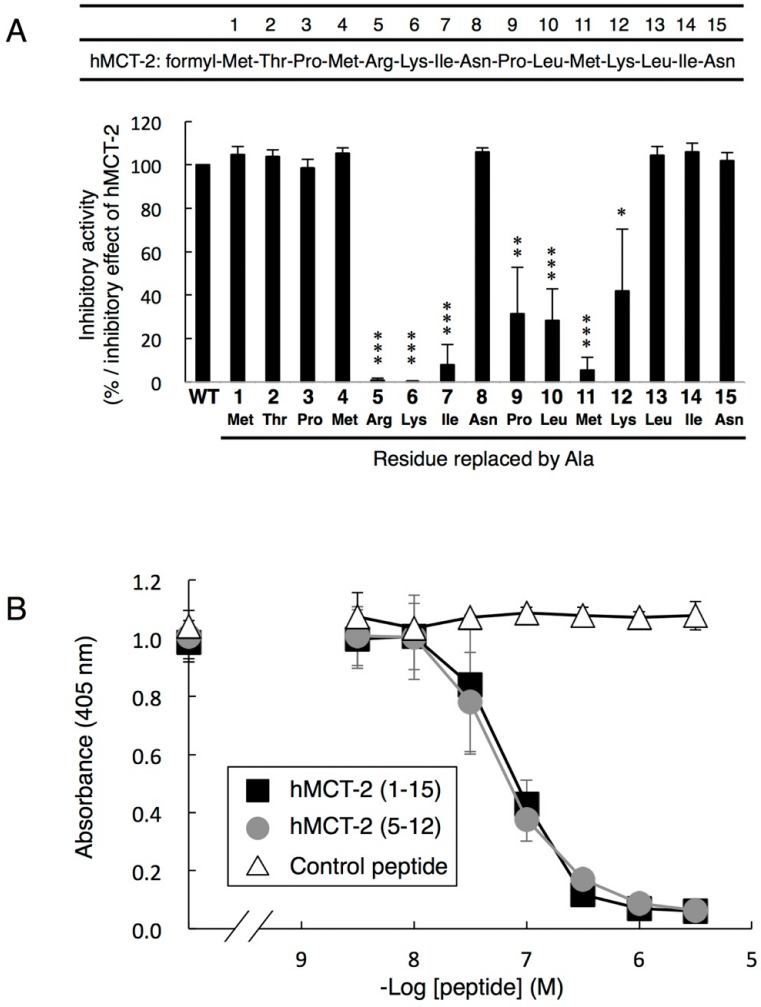

Fig. III-4. Mapping of the NhM2A5 epitope. (A) Alanine scanning of hMCT-2 by competition ELISA to find the important amino acid residues for binding NhM2A5. Inhibitory activity of hMCT-2 derivatives with single-residue Ala substitutions (1 μM) was expressed as the ratio to that of hMCT-2 on NhM2A5 binding (10 nM) to a 96-well plate with immobilized hMCT-2-EMCS-KLH. (B) Confirmation of minimum epitope region on hMCT-2 by competition ELISA using hMCT-2, hMCT-2 (5–12), and control peptide (porcine MCT-1). $*P < 0.05$, $**P < 0.01$, $***P < 0.001$ compared with inhibitory activity of hMCT-2. Each value is the mean ± SE of 3 separate experiments.

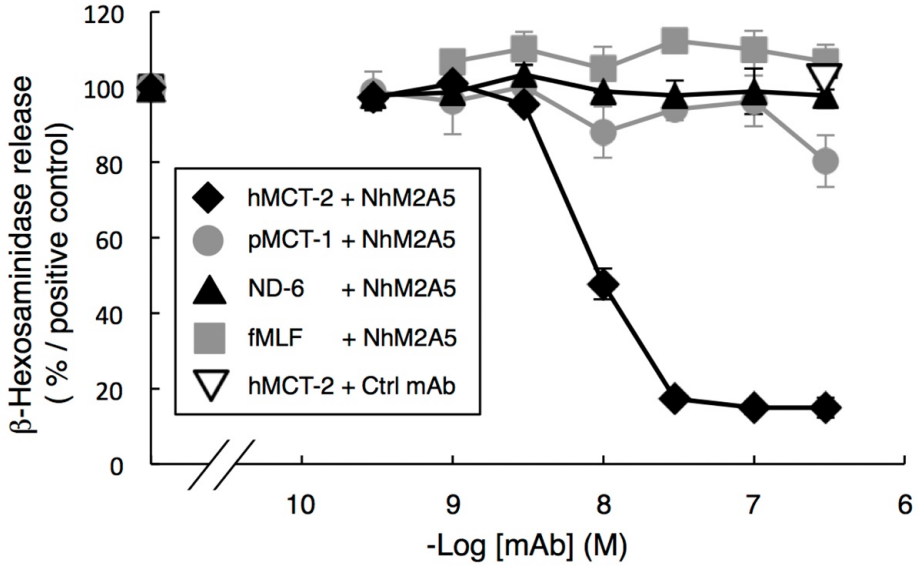

Fig. III-5. Inhibitory effect of NhM2A5 on hMCT-2-induced β-HA release in HL-60 cells differentiated into neutrophilic/granulocytic cells. β-HA release from the neutrophilic differentiated HL-60 cells stimulated by 50% of effective concentration of each peptide without mAbs was defined as positive control (100%). The β-HA release in the presence of various concentrations of NhM2A5 or isotype/subclass-matched control mAb (ctrl mAb) is expressed as a percentage. Experimental data are expressed as means ± SE of 4 separate experiments.

Chapter IV

General conclusions

MCT-1, MCT-2 and MCT-CYC have been identified from healthy porcine hearts as endogenous neutrophil-activating peptides and are the peptide fragments derived from mitochondrial cytochrome *c* oxidase subunit VIII, cytochrome *b*, and cytochrome *c*, respectively. These MCTs efficiently induce neutrophil migration and phagocytosis at nano-molar concentrations *in vitro*, but physiological production, localization and roles of MCTs remain unknown. There are two difficulties to investigate physiological functions of MCTs that hidden in mitochondrial proteins. One is that antibodies that bind to MCT sequences can not distinguish between MCTs and their parent proteins. Another reason is that silencing of gene that encoded MCTs inevitably affects the functions and expression levels of their parent proteins. In the present study, I tried to establish an alternative strategy for investigation of physiological and pathophysiological significance of MCTs.

Here, I attempted to generate monoclonal antibodies that bind to MCTs but not their parent proteins, and that specifically neutralize their bioactivities. Practically, I succeeded to obtain specific mAb, NM1B1 that bound to the region of positions 9 to 22 of MCT-1, and this mAb was shown to specifically prevent MCT-1-induced migration and phagocytosis of HL-60 cells differentiated into neutrophilic/granulocytic cells (*Chapter II*). I also acquired two anti-hMCT-2 mAbs with different features. Namely, NhM2A1 mAb bound to a cleavage site on hMCT-2 that is created by enzymatic hydrolysis of cytochrome *b*. This characteristic allows analysis of the production and localization of hMCT-2 because the mAb specifically binds to hMCT-2 but not to its parent protein, cytochrome *b*. Moreover, another mAb, NhM2A5 that bound to the

region of positions 5 to 12 of hMCT-2, was demonstrated to inhibit bioactivities of hMCT-2 specifically. This neutralizing mAb allows for investigation of its physiological roles without influencing the functions of its parent protein (*Chapter III*).

As described above, functional analysis of not only such MCTs but also various cryptides that hidden in protein structures had been presently very difficult. Generating specific mAbs against MCTs themselves and specific neutralizing mAbs against functions of MCTs contributes to investigation of their production, localization and functions in not only cellular levels but also in diseases including ischemia reperfusion injury, fulminant hepatic failure. Actually, the presence of MCT-2 in mtDAMPs has been shown by the immunoblot using NhM2A1 mAb, which specifically binds to MCT-2 very recently. Moreover, it was observed that intravenous injection of anti-MCT-1 neutralizing mAb, NM1B1, inhibited the accumulation of neutrophils into necrotic area of liver induced by a hepatic toxin, acetaminophen. Thus, this study will contribute to overcome the difficulties on elucidation of physiological significances of cryptides including MCTs.

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