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Inhibitory effects of C4a on chemoattractant and secretagogue functions of the other anaphylatoxins via Gi protein-adenylyl cyclase inhibition pathway in mast cells

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ABSTRACT

A recombinant complement anaphylatoxin, C4a, inhibited chemotaxis, respiratory burst and histamine release in mast cell-like HMC-1 cells that were treated with recombinant C5a anaphylatoxin. C4a also inhibited histamine release from HMC-1 cells that were induced by recombinant C3a. The inhibition of C5a- and C3a-induced leukocyte reactions by C4a was recapitulated in peripheral blood $CD133^+$ cell-derived differentiated mast cells. In HMC-1 cells, C4a inhibited cytoplasmic Ca^{2+} influx, an event that precedes anaphylatoxin-induced chemotactic and secretary responses. A conditioned medium of HMC-1 cells after shortly treated with C4a also inhibited the anaphylatoxin-induced Ca^{2+} influx even after removal of C4a, indicating that the effect of C4a is to liberate an autocrine inhibitor from the mast cells. The inhibitor secretion by C4a was prevented with pertussis toxin or with a phosphodiesterase inhibitor. Conversely, an adenylyl cyclase inhibitor reproduced the effect of C4a. C4a decreased the intracellular cyclic AMP concentration of HMC-1 cells, indicating that C4a elicited the Gi protein-adenylyl cyclase inhibition pathway. Neither C4a nor the conditioned medium, however, inhibited Ca^{2+} influx and respiratory burst in C5a- or C3a-stimulated peripheral neutrophils, suggesting that these cells lack this inhibitory system. Additionally, in HMC-1 cells, C4a did not inhibit Ca^{2+}-independent, Leu72Gln-C5a-stimulated chemotactic response. In agreement with this finding, C4a treatment inhibited ERK1/2 phosphorylation in HMC-1 cells stimulated with other anaphylatoxins but did not inhibit p38MAPK phosphorylation in cells stimulated with Leu72Gln-C5a. Taken together, these findings suggest that the autocrine inhibitory effect elicited by C4a is attributed to interruption of Ca^{2+}-dependent intracellular signaling pathway.

1. Introduction

Complement component 4 or C4, belongs to a distinct protein family that also includes C3 and C5. These molecules enable the foreign body-recognition components and the membrane attack components of the complement activation pathways to interact. These molecules also connect the complement system with various types of leukocytes. Chemical signaling to leukocytes, however, is not conveyed by the native molecules but by their fragments called anaphylatoxins, known as C3a, C4a and C5a. Anaphylatoxins are liberated from the N-terminal region of the parental protein α-chain. Anaphylatoxins are similar in terms of molecular structure. They are composed of 77 (C3a and C4a) or 74 (C5a) amino acid residues that are bridged intra-molecularly by 3 disulfide bonds [1]. Effects of C5a and C3a on leukocytes, especially neutrophils and mast cells, are well established. C5a exhibits chemoattractant and secretagogue effects on neutrophils, mast cells, monocytes and macrophages [2]. C3a exhibits secretagogue-only function in neutrophils and mast cells [3]. These effects of C5a and C3a are both associated with cytoplasmic Ca^{2+} influx [4]. Immuno-modulating capacities of C5a and C3a by dendritic cells have been also revealed [5,6], and these effects are mediated through the C5a receptor and the C3a receptor, respectively. Both the C5a and C3a receptors are hepta-helical transmembrane proteints coupled with pertussis toxin sensitive G proteins. In contrast to these anaphylatoxins, the receptor for C4a has not yet to be identified, and the function and role of C4a remains unclear.

We previously isolated C4a from inflammatory joint fluid of rheumatoid arthritis patients as the monocyte/macrophage migration inhibitory factor [7]. Later, we noticed that the inhibitory effect of C4a

Abbreviations: BSA, bovine serum albumin; ECL, enhanced chemiluminescence; *E. coli*, *Escherichia coli*; EDTA, ethylenediamine tetraacetic acid; ERK1/2, extracellular signal-regulated kinase1/2; FACS, fluorescence-activated cell sorting; FBS, fetal bovine serum; G protein, guanine nucleotide binding protein; HBSS, Hanks' balanced salt solution; HEPES, 2-[4-(hydroxyethyl)-1-piperazinyl] ethanesulfonicacid; HPLC, high performance liquid chromatography; L72Q-C5a, Leu72Gln-C5a; NADPH, nicotinamide adenine dinucleotide phosphate hydrogen; p38MAPK, p38 mitogen-activated protein kinase; PBS, phosphate-buffered saline; PCR, polymerase chain reaction; SDS- PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; WST-1, water-soluble monosodium tetrazolium salt.

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on C5a-induced monocyte migration was executed by an unidentified autocrine inhibitory molecule released by monocytes in response to C4a [8]. The inhibitory factor is a glycoprotein possessing molecular sizes around 17,000, and the amino-terminal of the protein moiety is somehow modified (Nishiura et al., unpublished data). Both C4a and the isolated inhibitory molecule failed to suppress chemotactic migration of neutrophils or T cells stimulated by C5a or interleukin-8, respectively [8]. We also reported that C4a inhibited C3a-induced cytoplasmic Ca^{2+} influx and respiratory burst in guinea pig peritoneal macrophages [9]. As such, we concluded that the inhibitory effect of C4a was restricted to monocytes/macrophages in the phagocytic leukocytes.

We recently determined that mast cell-like HMC-1 cells provide a useful model for the study of intracellular signal transduction pathways linking C5a receptor ligation to directional movement and histamine release [10]. In our preliminary study to identify the C4a receptor, we noticed that C4a interacted with HMC-1 cells to a significantly higher extent than it did with leukemia-derived cell lines such as HL-60, THP-1 and U937 cells. Mast cells have long been viewed as effector cells in acquired immunity, including type I allergy. It has also been shown that mast cells play prominent roles in innate immunity and in the linkage between innate and acquired immunity [11]. Mast cells are capable of engulfing bacteria, migrating to lymph nodes and presenting antigen information to T cells [12,13]. As such, mast cells appear to possess macrophage-like functions. In addition, an in vitro differentiation method of $CD133^+$ mast cell progenitors in peripheral blood within a relatively short culture period was recently reported [14]. Given these observations, we decided to examine if C4a treatment could inhibit C5a- or C3a-induced reactions in the HMC-1 mast cell line and in the in vitro differentiated primary mast cells. Recently, we found that C5a, mutated at Leu72 to Gln, maintained its chemoattractant capacity but lost its secretagogue capacity in the context of neutrophil stimulation. Additionally, we found that cytoplasmic Ca^{2+} influx was absent during the chemotactic response induced in neutrophils exposed to Leu72Gln-C5a [15]. These data suggest that while C5a and Leu72Gln-C5a both ligate the C5a receptor and induce chemotactic migration in neutrophils, the intracellular signaling pathways differ between these ligands. Thus, we also examined if Leu72Gln-C5a was able to attract HMC-1 cells and if C4a treatment could inhibit this process. Our data strongly suggest that C4a exerts inhibitory effects, via the autocrine inhibitor release, on mast cells and on monocytes or macrophages in response to exposure to the other anaphylatoxins by blocking authentic Ca^{2+}-dependent intracellular signaling pathways.

2. Materials and methods

2.1. Cells

HMC-1 cells were a kind gift from Dr. Joseph H. Butterfield of the Mayo Clinic, USA. Primary mast cell progenitors bearing CD133 were prepared from the mononuclear cell fraction in a Ficoll-Paque gradient of fresh venous blood (150 ml) of healthy donors using AC133 MicroBeads and LS + cell separation columns as described previously [14]. The $CD133^+$ cells were then cultured for 7 weeks being differentiated in the presence of stem cell factor, interleukin 6 and other reagents according to the method of Holm et al. [14]. Neutrophils were also isolated from heparinized human venous blood of healthy donors according to the method of Fernandez et al. [16] as described previously [7].

2.2. Reagents

Ficoll-Paque cell separation solution was purchased from GE Healthcare (Uppsala, Sweden). AC133 cell isolation kit and LS + cell separation columns were purchased from Miltenyi Biotech (Birgish Gladbach, Germany). Hanks' balanced salt solution (HBSS) was a product of Nissui Pharmaceutical CO. (Tokyo, Japan). Fura2-AM, water-soluble tetrazolium salt (WST-1) and HEPES buffer were purchased from Dojindo Laboratories (Kumamoto, Japan). Columns for high performance liquid column chromatography (HPLC) such as Hi-Trap™ Chelating HP, Hi-Trap™ Heparin HP and Hi-Trap™ Benzamidine FF (high sub) were obtained from Amersham Biosciences KK (Tokyo, Japan). Biotin N-hydroxysuccinimide ester, 4-(3-Butoxy-4-methoxybenzyl)-2-imidazolidinone (a phosphodiesterase inhibitor), , MDL-12,330A hydrochloride (an adenylyl cyclase inhibitor), pertussis toxin and bovine serum albumin (BSA) were purchased from Sigma Chemical (ST. Louis, MI). A multiwell chamber for chemotaxis assay was acquired from Neuro Probe (Bethesda, MD). Nucleopore filters were purchased from Nucleopore (Pleasant, CA). Rosetta-gami B (DE3) Lys-S *E. coli* and pET32a vector were purchased from Novagen (Darmstadt, Germany). DH5α *E. coli* and pBS(+)13 vector were obtained from Stratagene (San Diego, CA). Restriction enzymes and a RNA PCR kit were purchased from TaKaRa Biochemicals (Otsu, Japan). All other chemicals were obtained from Nacalai Tesque (Kyoto, Japan) or from Wako Pure Chemicals (Osaka, Japan) unless otherwise specified.

2.3. Preparation of C3a peptide fragment

A C3a peptide fragment with the amino acid sequence Leu-Arg-Arg-Gln-His-Ala-Arg-Ala-Ser-His-Leu-Gly-Leu-Ala-Arg (LRRQHARASHLGLAR) was synthesized by a conventional solid-phase method with fluorenylmethoxycarbonyl amino acid-resins. After synthesis, the peptides were separated from the resin with a trifluoroacetic acid-containing solvent. After extraction with water, the peptides were purified by preparative reverse-phase HPLC using a YMC C18 column (Φ 10 mm × 250 mm, Yamamura, Kyoto, Japan). The peptides thus prepared demonstrated single peaks with the absorbance at 220 nm as assessed by analytical reverse-phase HPLC using a Wako C18 column (Φ 4.6 × 150 mm) and exhibited the expected molecular mass in high resolution fast atom bombardment mass spectrometric analysis. After the peptides were frozen and dried, they were dissolved in sterilized phosphate-buffered saline (PBS, pH7.4) containing 1 mg/ml BSA and then used in our chemotaxis assay.

2.4. Preparation of recombinant anaphylatoxins

Recombinant C5a and Leu72Gln-C5a were prepared as described previously [15,17]. Recombinant C3a and C4a were prepared in a manner similar to C5a. In brief, C3a or C4a cDNA was prepared by RT-PCR using total mRNA derived from HepG2 cells and the primers 5′GAATTCATGTCCGTGCAGCTCAC3′ and 5′TTATTACCTGGCCAGGCCCA3′ or 5′ATGAATTCATCGTTGGAGGCCCGC3′ and 5′CGGATCCAACGTGAACTTCCAAAA3′, respectively. The confirmed nucleotide sequence of the PCR product was subcloned into the expression vector pET32a flanked by BamHI and EcoRI restriction sites, and each plasmid cDNA was transformed to DH5α competent cells. Rosetta-gami B (DE3) Lys-S transformed with each expression vector was cultured in LB medium containing ampicillin, chloramphenicol, kanamycin and tetracycline until the 600 nm absorbance of the bacterial suspension reached 0.6. Each suspension was mixed with 1 mM isopropyl 1-thio-β-D-galactoside and incubated for an additional 4 h. After centrifugation, the cultured *E. coli* cells were resuspended into 1/10 culture volume of 20 mM Tris–HCl containing 200 mM NaCl and 10 mM EDTA (pH 8.0). Bacteria were lysed by sonication in the presence of 1% Triton X-100. After centrifugation, the extracted recombinant proteins were separated using a Hi-Trap™ Chelating HP column pre-loaded with 100 mM $NiSO_4$ and the Hi-Trap™ SP HP column. The purity of each Trx-His-S-tag recombinant protein was inspected using polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate (SDS-PAGE) (data not shown). The recombinant proteins were dialyzed in PBS containing 1 mg/ml BSA and stored at −70 °C until use.