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Ancient origin of mast cells

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ABSTRACT

The sentinel roles of mammalian mast cells (MCs) in varied infections raised the question of their evolutionary origin. We discovered that the test cells in the sea squirt *Ciona intestinalis* morphologically and histochemically resembled cutaneous human MCs. Like the latter, *C. intestinalis* test cells stored histamine and varied heparin·serine protease complexes in their granules. Moreover, they exocytosed these preformed mediators when exposed to compound 48/80. In support of the histamine data, a *C. intestinalis*-derived cDNA was isolated that resembled that which encodes histidine decarboxylase in human MCs. Like heparin-expressing mammalian MCs, activated test cells produced prostaglandin D_2 and contained cDNAs that encode a protein that resembles the synthase needed for its biosynthesis in human MCs. The accumulated morphological, histochemical, biochemical, and molecular biology data suggest that the test cells in *C. intestinalis* are the counterparts of mammalian MCs that reside in varied connective tissues. The accumulated data point to an ancient origin of MCs that predates the emergence of the chordates >500 million years ago, well before the development of adaptive immunity. The remarkable conservation of MCs throughout evolution is consistent with their importance in innate immunity.

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1. Introduction

Mast cells (MCs) are important effector cells that participate in innate and adaptive immunity [1]. No human has been found who lacks MCs. Located at strategic sites within epithelial and mucosal surfaces, MCs perform sentinel roles in combating numerous pathogens, in part, via their exocytosed heparin·protease complexes [2–5]. Human MCs and their progenitors are highly susceptible to M-tropic strains of HIV-1 [6,7], and the loss of HIV-1-infected MCs in the gastrointestinal tract and other tissue sites [8] contributes to the development of AIDS.

When activated by complement anaphylatoxins or by varied pathogen-derived products, mammalian MCs quickly release their granule (e.g., histamine [9] and heparin·serine protease complexes [10–14]) and lipid (e.g., prostaglandin D_2 [PGD_2] [15] and leukotriene C_4 [LTC_4] [16]) mediators to initiate the acute phase of the inflammatory response against the infectious organism. Given their central roles in innate and acquired immunity, surprisingly little is known concerning the origin and evolution of different polarized subsets of MCs and their granule and lipid mediators. MCs have been identified in zebrafish [17]. Nevertheless, no protostome has been shown that possesses cells that resemble any subset of MCs in mice and humans. We therefore looked for MC-like cells in *Ciona intestinalis* which is a member of the chordate lineage that gave rise to vertebrates [18].

2. Materials and methods

2.1. Histochemistry and ultrastructure of the test cells in C. intestinalis, and biochemical characterization of the heparin glycosaminoglycans present in their secretory granules

C. intestinalis were collected at the Marine Biological Laboratory in Woods Hole, Massachusetts. The test cells that surround the oocytes of this sea squirt were isolated for *in vitro* study by mild physical trauma of the liberated eggs, following by sedimentation of their dense granulated test cells. The heparin proteoglycans and glycosaminoglycans were isolated from lysed test cells using the zwittergent 3–12 detergent/$CsCl_2$ density-gradient method we previously developed for the isolation of proteoglycans and glycosaminoglycans from rodent and human MCs [19]. Employing the experimental procedures developed for analyzing mammalian heparin glycosaminoglycans [20], purified test cell-derived heparin

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was digested with heparitinases (Seikagaku Corp.) and the resulting disaccharides were subjected to high performance liquid chromatography. After chromatographic separation of the generated disaccharides, they were reacted with 2-cyanoacetoamide as a post-column reagent. They were identified and quantitated based on comparisons of their elution positions and peak heights with those of known amounts of standard disaccharides.

2.2. Release of enzymatically active serine proteases from activated C. intestinalis test cells

C. intestinalis test cells ($\sim 1 \times 10^6$ cells/ml) were activated by exposure to 0.2 mM compound 48/80 (Sigma–Aldrich), as previously described for heparin-expressing mouse MCs [21] and *Styela plicata* test cells [22]. The treated cells were subjected to electron microscopy, using standard methodology [23,24]. In other experiments, supernatants were collected from the compound 48/80-activated test cells. The presence of enzymatically active serine proteases in the supernatants was detected using 5 nM of the biotinylated probe Phe-Pro-Arg-chloromethylketone (PPACK, Santa Cruz Biotech.), employing the experimental protocol recommended by the manufacturer. PPACK binds irreversibly to the active sites of serine proteases [25,26]. Thus, the PPACK-labeled serine proteases in the test cells of *C. intestinalis* were separated by SDS–PAGE, transferred to Protran BA83 nitrocellulose membranes (Whatman), blocked in 5% nonfat milk for 1 h, and incubated for 3 h with horseradish peroxidase (HRP)-conjugated streptavidin which binds with high affinity to the biotin moiety of the biontinylated-PPACK probe. The treated blots were washed three times (10 min each) in phosphate-buffered saline containing 0.1% Tween 20. They were then developed in enhanced chemiluminescence (ECL) reagent (Millipore) for 2–5 min. Labeled serine proteases were detected by exposing the blots to Blue XB-1 film (Kodak).

2.3. Release of histamine from activated C. intestinalis test cells, and cloning of the putative C. intestinalis ortholog of human histidine decarboxylase (HDC)

The histamine enzyme-linked immunosorbent assay (ELISA) created by Bertin Pharma/SPI Bio and distributed by Cayman Chemical was used to measure histamine levels in lysates of enriched test cells, again using the manufacturer's protocol. This ELISA is based on the competition between unlabeled derivatized histamine and aceylcholinesterase that has been linked to histamine (defined as the tracer) for a mouse monoclonal antihistamine antibody that is bound to a 96-well plate.

HDC [27] participates in the biosynthesis of histamine in mammalian MCs. A previously created cDNA library [28] was used to isolate its putative *C. intestinalis* ortholog (CiHDC). A search of the Expressed Sequence Tags (ESTs) in the library revealed a clone (designated *cidg826g09*) whose partial nucleotide sequence resembled that of human and mouse HDC. Using a standard molecular biology approach, the nucleotide sequence of the entire coding domain of CiHDC was determined, as well as the amino acid sequence of its translated protein. Finally, a guinea pig polyclonal anti-HDC antibody (Thermo Scientific) that recognizes human, mouse, chicken, and amphibian HDC was used to detect the presence of the putative CiHDC protein in test cells. Test cells were lysed in SDS–PAGE loading buffer. The liberated proteins were denatured at 95 °C for 5 min, fractionated on a 10% NuPAGE gel (Invitrogen), and immunoblotted with the anti-HDC antibody.

2.4. Release of prostaglandin D_2 (PGD_2) from activated C. intestinalis test cells, and cloning of the putative C. intestinalis ortholog of human hematopoietic-type PGD_2 synthase (HPGDS)

To determine if *C. intestinalis* test cells have the ability to generate PGD_2 and/or LTC_4 upon cellular activation, $\sim 10^6$ cells were placed in 200 µl of Hank's buffer containing 2 mg/ml of bovine serum albumin, 5 µM calcium ionophore A23187 (Calbiochem), and 10 µM arachidonic acid. One hour latter, the reactions were terminated by the addition of 2 volumes of methanol. After centrifugation at 12,000*g* for 10 min, half of the supernatants were analyzed for LTC_4 by reverse phase-high performance liquid chromatography [29]. The other half were dried under reduced pressure using a rotoevaporator, resuspended in ELISA buffer, and evaluated for their PGD_2 content using an ELISA kit (Cayman Chemical).

Rat, mouse, and human HPGDS catalyze the conversion of prostaglandin H_2 to PGD_2 [30] in heparin-expressing mammalian MCs [15]. We queried the EST database to isolate its putative *C. intestinalis* ortholog (CiHPGDS). The nucleotide sequences of the *C. intestinalis* ESTs BW487468 and BW062636 revealed significant homology to the respective 5′ and 3′ ends of the human HPGDS transcript noted at GenBank accession NP_055300. The nucleotide sequences that encode the entire coding regions of allelic isoforms of CiHPGDS were next determined using a test cell-enriched mRNA preparation and primers that were based on the above *C. intestinalis* ESTs. To that end, total RNA (1 µg) from test cells was reverse transcribed into cDNAs using SuperScript II reverse transcriptase (Life Technologies) and random hexamers. The CiHPGDS cDNAs were amplified using high fidelity Pfu polymerase (Stratagene). The forward and reverse primers used were 5′-ATGCCAGTTTAC AAGTTATACTACTTC-3′ and 5′-TTACATATTTGTCTTTGGTCTTGTG G-3′, respectively. The polymerase-chain reaction (PCR) cycling condition consisted of 35 cycles of denaturation (95 °C, 15 s), annealing (55 °C, 15 s), and extension (70 °C, 1 min). The resulting PCR products (~600 bp) were separated on 1% agarose gels, purified, and inserted in the pCR2.1 TOPO cloning vector (Life Technologies). The entire inserts from multiple independent clones were subjected to DNA sequencing.

3. Results and discussion

3.1. Histochemistry and ultrastructure of the test cells C. intestinalis test cells, and biochemical characterization of their granular heparin·protease complexes

All mammalian MCs possess non-segmented nuclei [23] and become metachromatic when stained with toluidine blue [31]. We discovered that the test cells in *C. intestinalis* histochemically and morphologically resembled the MCs that reside in the skin and other connective tissues of mammals in that they contained non-segmented nuclei and electron-dense granules that became metachromatic when stained with toluidine blue (Fig. 1A).

The T-cell-independent polarized subset of MCs that constitutively reside in the peritoneal cavity, skin, and other connective tissues of mice preferentially store heparin in their granules [32,33]. *S. plicata* contains a heparin glycosaminoglycan that structurally resembles that in the latter population of mammalian MCs [22]. In this urochordate, heparin is released upon cellular activation. We discovered that the test cells in *C. intestinalis* also contain classical heparin (Fig. 1B).

3.2. Release of enzymatically active serine proteases from activated C. intestinalis test cells

Heparin is essential for the packaging of tetramer-forming tryptases and other serine proteases inside the secretory granules of

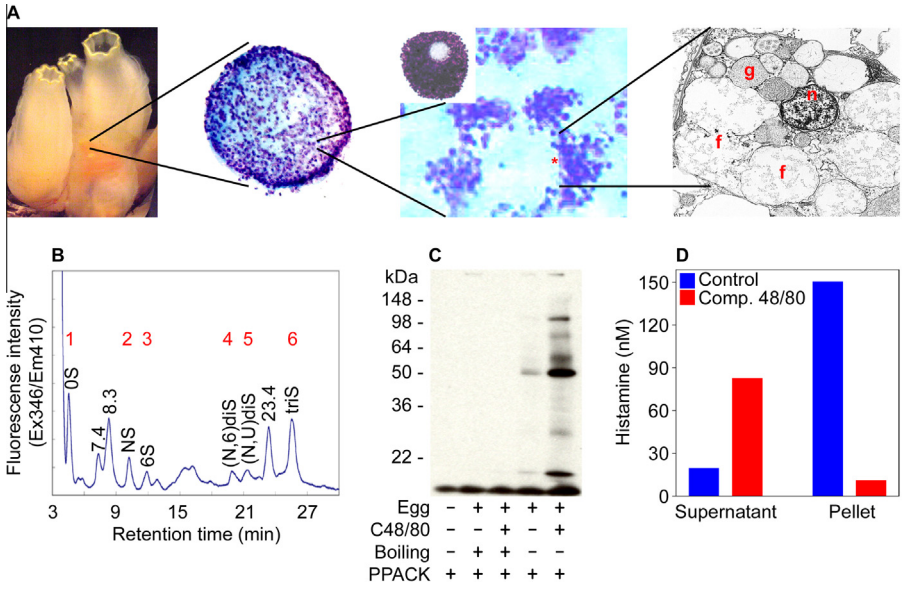

Fig. 1. The test cells that surround the oocyte in *C. intestinalis* resemble the MCs that reside in the skin and other connective tissues of humans and mice. (A) Depicted are increasing magnifications of *C. intestinalis* test cells. The cells in the middle panels were stained with toluidine blue. The circled area corresponds to a test cell. At the electron microscopic level, these cells contained a non-segmented nucleus (**n**) and electron-dense granules (**g**). When activated, many of these intracellular granules fused (**f**) before their contents were exocytosed analogous to activated mammalian MCs. (B) High performance liquid chromatography was carried out on the disaccharides obtained when purified test cell-derived heparin was incubated with heparitinases I–III. Peaks 1–6 correspond to the 0S, NS, 6S, (N,6)diS, (N,U)diS, and triS uronic acid-glucosamine disaccharides, respectively, based on their elution positions relative to that of known standards. The peak at 23.4 min also is present in a heparitinase digest of porcine heparin. Although its structure has not yet been deduced, almost certainly it is a disulfated disaccharide. The unknown peaks at 7.4 and 8.3 min most likely are monosulfated disaccharides. (C, D) Compound 48/80-treated test cell-enriched oocytes also exocytose enzymatically active serine proteases (C) and histamine (D).

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CiHDCL    1 MTEDNNVEVPRLGIEPEAFRLAAANMVDYVIKYYCDVDKRQTFSDVKPGFMRALLPESPPDRPESWQEVFS
                        |||  |      ||||    |   |  |     || ||  || |||| |  | ||    |
hHDC      1              MMEPEEYRERGREMVDYICQYLSTVRERRVTPDVQPGYLRAQLPESAPEDPDSWDSIFG

CiHDCL   72 DIERIVMDGMTHWQSPGFFSYYPSSASYPSMLADMLCSGVPCIGFSWASSPSCTELETVMMDWLGKAIGLP
            ||||| | |  |||||     |||    | || | |||      | || ||||| |||||    |||| |  |||
hHDC     60 DIERIIMPGVVHWQSPHMHAYYPALTSWPSLLGDMLADAINCLGFTWASSPACTELEMNVMDWLAKMLGLP

CiHDCL  143 ECFIHGGHGP-GGGVIQGTASEATLVALIAARSKTIRRELSRDPNQRTHDIVGRMVAYTSQCSHSSVERAG
            | | |       |||| |  | || || || |||    |         |          | ||| |    |||||  ||
hHDC    131 EHFLHHHPSSQGGGVLQSTVSESTLIALLAARKNKILEMKTSEPDADESCLNARLVAYASDQAHSSVEKAG

CiHDCL  213 LLSLVEVRRLPVKDDGALEGGVLKEAVLEDRKAGRIPMFVCVTIGTTSCCTFDDLEGIGKTCETEDIWCHV
            | |||     ||| |    | |   |   |   ||    |   |  || | |||   | || |    |   |   |  | |
hHDC    202 LISLVKMKFLPVDDNFSLRGEALQKAIEEDKQRGLVPVFVCATLGTTGVCAFDCLSELGPICAREGLWLHI

CiHDCL  284 DAAYAGAALVCPEFRFICKGIERATSFNFNPHKWLMVQFDCSAMWVRDSTDLINSAEVNPLYLRHNTESAT
            |||||| |  |||||    |||| | || ||| || || |||    || |    |      ||| ||||
hHDC    273 DAAYAGTAFLCPEFRGFLKGIEYADSFTFNPSKWMMVHFDCTGFWVKDKYKLQQTFSVNPIYLRHANSGVA

CiHDCL  355 IDYRHWQIPLGRRFRSLKLWFVLRMVGVEGLRSHIRRGVREAKHLEELVRCDERFEVLFPVILGLVCIKLK
             |  |||||| ||||| |||||  |   ||   |  | |  |    ||  | ||| |  ||       ||||    ||
hHDC    344 TDFMHWQIPLSRRFRSVKLWFVIRSFGVKNLQAHVRHGTEMAKYFESLVRNDPSFEIPAKRHLGLVVFRLK

CiHDCL  426 RPGSSLEDENDLNERLYDKIHEDRRIFIVPATLNGVYFIRICTGSTHCSIEQVNKCWQVITEMAGEL  492
            |        | | |            | |  |||       ||     |            |  |    |
hHDC    415 GP-------NCLTENVLKEIAKAGRLFLIPATIQDKLIIRFTVTSQFTTRDDILRDWNLIRDAATLI  474
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Fig. 2. Isolation and characterization of a *C. intestinalis* transcript that encodes a HDC-like protein. A *C. intestinalis* cDNA was isolated (see GenBank accession number EF125183) that encoded a novel 492-mer protein that was ~50% identical to residues 1–474 of human histidine decarboxylase (**hHDC**). Additional information on this cDNA (accession number *cidg826g09*) can be found at http://ghost.zool.kyoto-u.ac.jp/. Shown is a comparison of the *C. intestinalis* HDC-like protein (**CiHDCL**) and **hHDC**. Using an expression/site-directed mutagenesis approach, two regions (corresponding to underlined residues 60–123 and 273–313 in **hHDC**) and seven amino acids (i.e., Y^{80}, A^{83}, D^{273}, N^{302}, K^{305}, Y^{334}, and R^{336}) have been identified in **hHDC** that are important for the enzymatic activity of this decarboxylase. **CiHDCL** possesses those regions as well as the amino acids that are essential for histamine biosynthesis. **hHDC** is abundant in the gastric mucosa of humans as well as their tissue MCs. The fact that the **CiHDCL** cDNA was isolated from the sea squirt's digestive gland is further evidence that it is the likely ortholog of **hHDC**. Only one **CiHDCL** EST was present in our library of ~600,000 *C. intestinalis* ESTs. Thus, **CiHDCL** is a highly restricted transcript in *C. intestinalis* as is **HDC** in humans.

cutaneous mouse and human MCs [33,34], and the exocytosed heparin·tryptase complexes are essential for combating *Klebsiella pneumonia* [3] and *Trichinella spiralis* [5]. Mammalian MCs are heterogeneous and compound 48/80 is a potent secretagogue of the subset of MCs that reside in the peritoneal cavity, skin, and connective tissues of mice due to the drug's ability to bind to varied members of the MAS family of G-protein coupled receptors [35,36]. *C. intestinalis* contain a large repertoire of genes (~170) that encode various classes of the G-protein coupled receptors [37]. When our enriched preparations of *C. intestinalis* test cells were exposed to compound 48/80, the cell's granules quickly fused intracellularly. Their contents were then released *en mass* into the extracellular environment.

As occurs in activated mammalian MCs, we detected substantial amounts of enzymatically active serine proteases in the resulting supernatants of the compound 48/80-treated cells using PPACK (Fig. 1C). hTryptase-β [12] and mMCP-6 [13] are tetramer-forming tryptases stored in abundance in the secretory granules of human and mouse MCs, respectively, ionically bound to heparin [33]. Through genome sequence comparison, we identified numerous *C. intestinalis* genes and transcripts that encode proteins that are 32–38% identical to mMCP-6 and hTryptase-β (e.g., XP_002130273, XP_002120017, XP_002126930, and XP_002129039), suggesting that one or more of these tryptic-like serine proteases resides in the heparin-rich secretory granules of the sea squirt's test cells.

3.3. Release of histamine from activated C. intestinalis test cells, and cloning of the putative C. intestinalis ortholog of HDC

While mammalian MCs also store substantial amounts of histamine in their secretory granules, Dehal and coworkers failed to identify a histidine decarboxylase-like (*CiHDCL*) gene in their initial draft of the *C. intestinalis* genome [38]. While these unanticipated data led to the tentative conclusion that sea squirts lack histamine and therefore no MCs, we discovered that histamine was exocytosed from our compound 48/80-treated test cells (Fig. 1D). In support of these data, Cavalcante and coworkers [39] discovered that the corresponding test cells in *S. plicata* also contained histamine. To resolve these discrepancies, we cloned a novel cDNA that encodes a previously uncharacterized *C. intestinalis* protein (Fig. 2) that remarkably was ~50% identical to human HDC [27]. The nucleotide sequence of the CiHDC-like cDNA was deposited in GenBank database (accession number EF125183). We also identified an immunoreactive protein in lysates of the test cell-enriched oocytes of *C. intestinalis* that cross-reacted with a polyclonal antibody that recognizes mammalian and amphibian HDC (data not shown).

3.4. Release of PGD_2 from activated C. intestinalis test cells, and cloning of the putative C. intestinalis ortholog of human HPGDS

Activated rodent heparin$^+$ MCs preferentially metabolize arachidonic acid to PGD_2 [15], whereas activated rodent heparin$^-$ MCs produce primarily LTC_4 [16]. A search of the *C. intestinalis* genome and EST database revealed HPGDS-like genes, but no LTC_4 synthase-like gene. Using primers that reside in the 5′ and 3′ ends of one of these HPGDS-like genes, we isolated full-length CiHPGDS-like transcripts in a test cell-enriched mRNA preparation. Cloning and sequencing of the ~600-bp PCR products revealed three closely related cDNAs (GenBank accession numbers DQ789056, DQ789057, and DQ789058) that encode isoforms of the 197-mer CiHPGDS whose translated products differ only at amino acids 28, 57, 116, and 166. These cDNAs likely represent allelic isoforms of the enzyme since the genome of *C. intestinalis* is highly polymorphic [40]. At the protein levels, CiHPGDS is 38% and 39% identical to mouse and human HPGDS, respectively. Consistent with our DNA and RNA data, substantial amounts of PGD_2 (but not LTC_4) were generated in a time-dependent manner when *C. intestinalis* test cells were given arachidonic acid and calcium ionophore *ex vivo* (data not shown).

Our morphological, histochemical, biochemical, and molecular biology data suggest that the PGD_2 pathway of arachidonic acid metabolism developed in evolution before the LTC_4 pathway, that heparin evolved to store histamine and varied enzymatically active serine proteases in the MC's secretory granules, and that heparin-expressing MCs appeared >500 million years ago before the development of adaptive immunity.

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