Histidine Decarboxylase Expression in Mouse Mast Cell Line P815 Is Induced by Mouse Peritoneal Cavity Incubation*

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Phenotype of P815 mouse mast cells changes markedly during culture in the peritoneal cavity of syngenic BDF1 mice. The cells, cultured for 1 week in the peritoneal cavity of syngenic BDF1 mice, proliferate and express high levels of l-histidine decarboxylase (HDC) and mouse mast cell protease (MMCP)-6 mRNAs, indicating the ability of P815 cells to differentiate toward mature connective tissue mast cells. Peritoneal fluid aspirated from P815-inoculated BDF1 mouse and added to cultured P815 cells in vitro was also found to induce HDC mRNA expression, suggesting that at least some of the humoral factors in the peritoneal fluid induce HDC mRNA transcription. Among the erythroid transcription factors, P815 cells expressed GATA-2 but not GATA-1 mRNA before and after the intraperitoneal incubation. In contrast, the expression of NF-E2 subunit p45 disappeared, while expression of subunit mafK was markedly reduced after incubation. Cotransfection assays using HDC-luciferase reporter and p45 and/or mafK expression constructs showed that NF-E2 affects the transactivation of HDC gene. These results suggest that NF-E2 is also an important transcription factor in mast cell differentiation.

Precursor cells derived from multipotential stem cells differentiate along defined cell lineages in vivo to become mature cells. Mast cells are derived from progenitor cells in the bone marrow and show a characteristic differentiation profile. Differentiation is completed after these cells reach peripheral tissues such as skin, peritoneal cavity, and bronchial submucosa (1). Mouse mast cells are classified into at least two subtypes, i.e., mucosal and connective tissue mast cells (MMC and CTMC), both of which are derived from common precursor cells (2). Mature mast cells retain more intracellular granules containing mast cell-specific proteases than undifferentiated mast cells (3–5). Whereas the proteases in MMC are primarily MMCP-1 (6) and MMCP-2 (7), in CTMC the main granular proteases are MC-CPA (8), MMCP-4 (9), MMCP-5 (10), and MMCP-6 (5, 11). The expression of each mast cell protease changes during differentiation. Therefore, examination of the expression of granular proteases is one way to assess the stage of differentiation and the subtypes of mast cells.

Expression of genes conferring certain specific phenotypes during development and differentiation processes is often controlled by lineage-specific transcription factors. For instance, GATA factors and NF-E2 are known to play important roles in the expression of erythroid genes (12). Targeted disruption of mouse GATA-1 and GATA-2 genes results in impaired maturation of erythroid cells and loss of hematopoietic stem cells, respectively (13–17). Furthermore, mast cell-specific genes MC-CPA and IL-4 were also activated by GATA factors (18, 19). Therefore, it is suggested that GATA factors are not only expressed but important for the function of mast cells. NF-E2 consists of a hematopoietic lineage-specific p45 subunit and a broadly expressed p18 subunit (20, 21). Recently, the p18 subunit was identified as the small Maf family protein MafK (22). These transcription factors are reported to be important for erythroid gene expression and are also found in mast cells (18, 19, 23, 24). For this reason, we are interested to find whether NF-E2 also plays a role in mast cell differentiation.

Characterization of erythroid transcription factors involved in the establishment of the mast cell lineage could provide a clue as to how the same transcription factors activate a distinct set of genes in erythroid, mast cell, and megakaryocyte lineages. In contrast to the availability of many cell differentiation systems for erythroid lineage, only a limited number of in vitro mast cell differentiation systems are available (25–27). Consequently, the differentiation process of mast cells is not well characterized in the relation to those transcription factors. In this regard, we noticed that mouse mast cell line P815 may provide a good model system to study mast cell differentiation. The P815 cell line was established from a mastocytoma in a DBB2 mouse and can proliferate in the peritoneal cavity of the syngenic mouse (28). We observed that P815 cells accumulate large quantities of histidine decarboxylase (HDC (EC 4.1.1.22)) during the intraperitoneal culture. As this enzyme catalyzes the synthesis of histamine, a characteristic monoamine in mast cells, and basophils in hematopoietic cells, we hypothesized that changes in P815 cells during intraperitoneal culture should mimic the differentiation process of mast cells in vivo.

We also observed that P815 cells expressed a high level of mast cell granular protease MMCP-6 after 1 week of intraperitoneal culture. In contrast, the NF-E2 subunits p45 and, to a lesser extent, mafK were markedly down-regulated 1 week...
after inoculation. Finally, to assess the direct effect of p45 and/or mafK on HDC gene expression, we cotransfected p45 and/or mafK expression constructs with the HDC-reporter construct. The results demonstrated that p45 and/or MafK regulate the transactivation of the HDC gene.

**Materials and Methods**

**Cell Lines**—Mouse mast cell line P815 was obtained from Dr. M. Potter (28) and maintained in RPMI 1640 medium containing 10% fetal bovine serum. Intraperitoneal Induction—A suspension of 2 × 10⁶ P815 cells was injected into the peritoneal cavity of BDF1 mice. One week later, the abdominal wall of the mice was expanded with increased numbers of peritoneal cells. The P815 cells were cultured in RPMI 1640 medium with a final concentration of 50% peritoneal fluid. After incubation, and the mouse died approximately 10 days after inoculation. Suspensions of 2 × 10⁶ peritoneal cells were injected into another mouse (2nd injection) to maintain these cells.

**Measurement of Histamine Content**—Histamine content of P815 cells was measured by using an HPLC fluorometric system as described (29). Fluorescence was measured with a fluoromonitor (F-1100, Hitachi, Tokyo) using excitation and emission wavelengths of 360 and 450 nm, respectively.

**HDC Activity Assay**—HDC activity was measured as described by the method as described (30). P815 cells were suspended in a solution (HDC reaction buffer) of 0.1 M potassium phosphate buffer (pH 6.8) containing 0.2 mM diithiothreitol, 0.1 mM pyridoxal 5'-phosphate, 1% polyethylene glycol (Mr = 3000) and 100 μg/ml phenylmethylsulfonyl fluoride, sonicated for 20 s, and centrifuged at 10,000 × g for 15 min at 4°C. An aliquot of 50 μl of the supernatant was injected into the HPLC system in which histamine was separated on a cation-exchanger TSK SP-25W (6 mm inside diameter × 15 cm, Tosoh, Tokyo) column and automatically mixed with d-phaladahyde in alkaline conditions. Fluorescence was measured with a fluorometer (F-1100, Hitachi, Tokyo) using excitation and emission wavelengths of 360 and 450 nm, respectively.

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**Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR) Analysis**—One μg of total RNA was reverse-transcribed using 200 units of SuperScript II reverse transcriptase (Life Technologies) in a solution containing 0.5 μM dNTP mix and 10 μg/ml random oligonucleotide primers in a total volume of 20 μl. For preparation of the cDNA for MMCP-6, total RNA was first treated with DNase I (20 μl/mg) to digest the genomic DNA because an extra signal corresponding to genomic DNA was observed on amplification. All PCR reactions were carried out in a total volume of 30 μl of solution containing 1 μl of cDNA product, 0.75 unit of Ex Taq polymerase (Takara Suyzo, Ohtsu, Japan), 0.2 mM dNTP mixture, and 10 μM specific sets of primers (Table 5) using a thermal cycler (JPT9900, Takara Suyzo, Ohtsu, Japan). Cycling condition is 40 cycles, 94°C for 1 min, 50°C for 1 min, and 72°C for 2 min. The products were separated by electrophoresis in 2% agarose gel and stained with ethidium bromide.

**RNA Blot Hybridization Analysis**—Total RNA was extracted by the acid guanidium thiocyanate/phenol chloroform extraction method (34). Samples of 15 μg of total RNA were subjected to electrophoresis in 1.0% agarose/formaldehyde gel. After electrophoresis, the RNA samples were transferred to nylon filters (Hybond-N, Amersham) with 20 × SSC. 32P-Labeled specific cDNA probes were hybridized with the filter in hybridizing solution (50% formamide, 6 × SSC, 5 × Denhardt's solution, 0.5% SDS, and 100 μg/ml salmon sperm) at 42°C overnight. After two washings with 2 × SSC and 1% SDS and then twice with 0.2 × SSC and 0.1% SDS at 42°C, the filter was exposed to Kodak X-Omat film at −80°C with an intensifying screen.

**In Situ Hybridization of P815 Cells**—P815 cells were washed and suspended in PBS to a final 2 × 10⁶ cells/ml. 100 μl of the suspension was centrifuged in the cytospin (Shandon, Runcorn) at 700 rpm for 5 min. Cells were fixed in 4% paraformaldehyde/Tris-buffered saline (TBS) for 30 min followed by proteinase K (20 μg/ml) treatment at 37°C for 30 min. After preparation of the cytospins, in situ hybridization reactions were carried out utilizing a digoxigenin-alkaline phosphatase system (Boehringer Mannheim, Mannheim, Germany) as described previously (35) with minor modifications. Briefly, digoxigenin-labeled RNA probes were generated by transcribing linearized pBS HDC7 with T7 RNA polymerase. pBS HDC7 contains the entire coding region of HDC cDNA (2.0 kilobases). Transcribed RNA were hydrolyzed with 0.1 M NaHCO₃ to an average size of 150 bases. After hybridization and alkaline phosphatase reaction with nitro blue tetrazolium salt, the samples were counterstained by Safranine O (Wako, Osaka).

**Cotransfection of QT6 Cells with HDC-luciferase and p45 and/or MafK Expression Vectors**—The construction of eukaryotic expression plasmids of mouse MafK (pEFMafK) and p45 NF-E2 (pEFp45) were described previously (21, 22). The luciferase reporter plasmid pGLm-1099 was constructed as follows. The upstream primer of PCR reaction was designed from the nucleotide sequence −1099 to −1073 of the mouse HDC gene and had an XbaI site in its 5' end. The downstream primer was designed from the nucleotide sequence +65 to +91 and had an SphI site in its 5' end. HDC phage clone was amplified and digested with XbaI and SphI for subcloning into the XbaI-SphI site of pUC00CAT (36). This plasmid was digested again with BamHI and HindIII, and was subcloned into the BglII-HindIII site of pGL2-basic (Promega).

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transfection. Cells were transfected by the calcium phosphate precipitation method (38) with 1.0 μg of pGLm-1099, 1.0 μg of pENL as an internal control, various combinations of effector plasmids, e.g. pEFm-MafK, pEPp45, and pEF-BassHIII to equalize the total amount of DNA (4.0 μg/16-mm dish). 12 h after transfection, cells were washed with phosphate-buffered saline (PBS), fed with fresh Dulbecco’s modified Eagle’s medium with 10% fetal bovine serum, and incubated for another 24 h. Preparation of cell lysates and luciferase assay were carried out using a Luciferase Assay Kit (TOYO-Inki Japan, Tokyo) and a Biolumat luminometer (Berthold Japan, Tokyo). β-Galactosidase activities in cell lysates were assayed as described (38). Data were expressed as the means of relative luciferase activities normalized for β-galactosidase activities. Five independent transfections were carried out, each in duplicate.

RESULTS

**P815 Cells after Intraperitoneal Culture Exhibit Increased Histamine Content and HDC Activity**—Mouse mast cell line P815 was established from a mastocytoma in a DBf/2 mouse and proliferates in the peritoneal cavity of syngenic mouse (28). In our observation, 1 week after inoculation of 2 × 10^6 P815 cells into the peritoneal cavity of syngenic BDF1 mouse, the abdominal wall was expanded with proliferating P815 cells and viscous peritoneal fluid. The number of P815 cells recovered from the peritoneal cavity reached 2.2 (± 0.43) × 10^8 per mouse.

We first examined changes in histamine content in P815 cells before and after intraperitoneal culture. The average histamine content per cell increased about 14-fold in 1 week (Fig. 1A). Activity of histidine decarboxylase (HDC) also increased about 42-fold (Fig. 1B). To assess whether the resident peritoneal cells contribute to the increases of histamine content and HDC activity in these experiments, the peritoneal cavities of five control BDF1 mice were lavaged with RPMI 1640 medium, and the histamine content and HDC activity were measured. The average histamine content of the peritoneal resident cells was 3.3 nmol/10^6 cells, and the recovery of cells was 7.2 × 10^6 per mouse. Therefore, the average total histamine content was 29.8 nmol per mouse. By subtraction of the histamine content of resident cells from that of inoculated peritoneal cells, the amount of histamine in the proliferated P815 cells was calculated to be 542 pmol/10^6 cells, assuming that there was no proliferation of the resident cells or immigration of cells into the peritoneal cavity. This level is about 11.3-fold higher than that of uninoculated P815 cells. Similar analysis showed that the HDC activity after intraperitoneal culture increased 41-fold over that of uninoculated P815 cells. Therefore, histamine synthesis was supposed to increase in P815 cells after intraperitoneal incubation.

**In Situ Hybridization of P815 Cells with HDC mRNA as the Probe**—After recovering the cells from the peritoneal cavity, the cells were stained to see the expression of HDC mRNA in each cell (Fig. 2A). Almost all cells expressed the HDC mRNA although the intensity of staining was not even but heterologous. In contrast, the control P815 cells before intraperitoneal culture were not stained (Fig. 2B). The cells did not acquire any granules positively stained with berberine sulfate which is known to bind to heparin even after the intraperitoneal culture (data not shown). This suggests P815 cells partially matured along mast cell lineage but do not reach until the terminal differentiation.

**Expression of HDC and Mast Cell Specific Secretory Granule Proteases in P815 Cells during Intraperitoneal Culture**—We next examined changes of HDC and secretory granule protease mRNA levels in P815 cells during culture in vitro. The expression of HDC mRNA increased during the first week of intraperitoneal culture and the level did not change after successive transfer into other mice (Fig. 3). Expression of MMCP-4 and -7 mRNAs was never detected in P815 cells throughout the study (data not shown), MMCP-6 was found to be markedly induced (Fig. 3), and the level of MC-CPA did not change significantly (Fig. 3). These data suggest that P815 cells are differentiating toward the mature CTMC under intraperitoneal culture conditions.

**Culture of P815 Cells in Vitro with Peritoneal Fluid from BDF1 Mouse Harboring P815 Cells**—As shown above, P815 cells differentiated and expressed HDC mRNA 1 week after intraperitoneal culture. To determine whether this process is regulated by humoral factor(s), factor(s) bound to the extracellular matrix, or cell-to-cell contact in the cavity, we collected peritoneal fluid from BDF1 mouse inoculated with P815 cells for 1 week. The fluid was filtered, and the filtrate was added to the culture medium of P815 cells in vitro to assess the expression of HDC mRNA. By day 4, P815 cells expressed HDC mRNA, whereas P815 cells cultured with the peritoneal lavage fluid from uninoculated BDF1 mice did not express HDC mRNA (Fig. 4). These results suggest that humoral factor(s) produced by the peritoneal cavity incubation of P815 cells induces ex-
and a control without RNA (mRNA expressions of P815 cells cultured in RPMI 1640 medium with specific primers of mouse HDC cDNA (Table I). The HDC amplified for comparison. As a positive control, mouse HDC cDNA plasmid was loaded in each lane (data not shown).

Next, we compared the level of mRNA expression of four erythroid transcription factors before and during intraperitoneal culture.

Reversibility of HDC mRNA Expression in P815 Cells—To examine whether activation of HDC in P815 cells under peritoneal incubation is reversible, we aspirated peritoneal cells from the peritoneal cavity and cultured them in RPMI 1640 medium for 7 days. The cells proliferated well, and the expression of HDC mRNA started to decrease after 3 days and became undetectable after 7 days (Fig. 5). Therefore, this finding suggests that P815 cells reverted the phenotypic feature of the cells in RPMI medium not to express HDC mRNA again.

Erythroid transcription factor NF-E2 is a heterodimer of basic-leucine zipper proteins composed of small MafK and large p45 subunits. mafK and p45 mRNAs were both found in the control P815 cells (Fig. 6). However, p45 mRNA was not detectable in these cells after 1 week of intraperitoneal culture. The level of mafK mRNA was also decreased, but the mRNA was still detectable after intraperitoneal culture. The expression of p45 NF-E2 mRNA was further analyzed by blot hybridization using poly(A)+ RNA (data not shown). The results confirmed that mRNA of p45 disappeared after intraperitoneal culture. No mafK mRNA expression was detectable even by using poly(A)− RNA (data not shown). In summary, during in vivo differentiation of P815, the expressions of GATA-1 and GATA-2 mRNA did not change, while NF-E2 expression decreased significantly. The result suggests that the down-regulation of NF-E2 expression may be an important event during differentiation of mast cells.

Regulation of 5′-Flanking Region of HDC Gene by MafK and p45—To assess the effects of MafK and p45 on transcriptional activity of the HDC gene, we cotransfected p45 and MafK expression constructs with a reporter construct containing the HDC upstream region. Reporter plasmid pGL-m1099 contains a 1.2-kilobase fragment of the mouse HDC upstream region from −1099 base to +91 base placed 5′ multiple cloning site of pGL2-basic. +91 base is located just upstream of the initiation site of the coding region. Various combinations of reporter and effector plasmids were transfected into QT6 cells. Cotransfection of p45 expression plasmid (pEFp45) with HDC-reporter plasmid (pGL-m1099) resulted in suppressed reporter gene activity within transfected cells (Fig. 7). On the contrary, the luciferase activity of pGL-m1099 was enhanced by MafK expression plasmid (pEFmMafK). When transfected with pEFp45 and pEFmMafK, the luciferase activity of P815 cells still decreased, suggesting that p45 dominantly suppress the transactivation of HDC gene.
but HDC mRNA expression during differentiation has not yet been reported. Our data indicate that HDC mRNA expression also increases during differentiation of P815 mast cells in vivo. Since it was proved by in situ hybridization that almost all of the cells recovered from the peritoneal cavity expressed HDC mRNA, it seemed to be unlikely that the emergence of the HDC and MMCP-6 mRNA only contributed to the resident mast cells or macrophage, but the intraperitoneally inoculated P815 themselves changed their phenotypic feature to express HDC mRNA. The number of P815 cells recovered from the peritoneal cavity reached 2.2 (± 0.43) x 10^6 per mouse 1 week after the inoculation which will be sufficient for many biochemical and molecular biological studies. Thus, this in vivo culture system may provide a versatile mast cell culture system through which the differentiation process of CTMC can be more carefully controlled and analyzed.

To determine whether the induction of HDC mRNA expression is regulated by transferable humoral factor(s), HDC mRNA expression in P815 cells was assessed after 4 days of incubation with the filtered peritoneal fluid. Some transferable and humoral factor(s) from the host BDF1 mouse is strongly suggested to induce the differentiation of P815 cells, as in vitro cultured P815 cells changed to express HDC mRNA under the culture condition with the filtered peritoneal fluid. This change of P815 cells was found to be reversible, since the HDC mRNA expression in P815 cells disappeared when P815 cells recovered from the peritoneal cavity were cultured in normal RPMI 1640 medium with 10% fetal bovine serum for 7 days. There are many cytokines known to induce the differentiation of mast cells, such as IL-3 (39), IL-9 (27), nerve growth factor (25), or stem cell factor (40). However, IL-3, nerve growth factor, and stem cell factor are unlikely to induce the change of P815

Fig. 6. GATA-1, GATA-2, p45, and mafK mRNA expressions in P815 cells during intraperitoneal culture. Total RNA isolated from P815 cells obtained before (lane C) and 1 week after (lane 1) inoculation were analyzed by RT-PCR amplification as described under “Materials and Methods” with specific primers of mouse GATA-1, GATA-2, p45, and mafK cDNA (Table I). The mouse erythroleukemia cell line (MEL) expresses GATA-1 mRNA but no GATA-2 mRNA (H. Harigae, N. Suwabe, H. Ohta, H. Ohtani, L. Gu, Z. Yang, T. Suda, F.-Y. Tsai, S. H. Orkin, J. D. Engel, and M. Yamamoto, unpublished data), and so this lane was prepared as a control (lane MEL). As a positive PCR reaction control, mouse GATA-1 and GATA-2 cDNA plasmids were amplified (lane cDNA). The β-actin gene was used as control for evaluation of the amount of cDNA synthesized.

indipendently because by externally adding these factors we are not able to detect any induction of HDC expression in P815 cells (data not shown). Although the possibility remains that these factors act synergistically and/or sequentially on P815 cells, which has been reported for bone marrow-derived mast cells (5).

MMCP-6 is a mouse trypstase reported to be transcribed in CTMC but not in MMC (11). In concordance with this report, microphthalmia mutant (mi/mi) mice that lack CTMC express low levels of MMCP-6 in the skin, but normal amounts of MC-CPA (41). During incubation in the peritoneal cavity, P815 cells acquired the expression of MMCP-6 mRNA. Therefore, P815 cells appeared to have differentiated toward CTMC during intraperitoneal culture. On the other hand, the expression of MC-CPA mRNA in P815 cells was constant throughout the study, suggesting that the expression of MC-CPA mRNA precedes that of MMCP-6 mRNA during differentiation. P815 cells cultured in RPMI 1640 medium do not express MMCP-2 (42), a marker of MMC (7). Together with our present observations, this suggests that the maturational stage of P815 cells before intraperitoneal culture is similar to that of immature mast cells before divergence to MMC and CTMC. MMCP-4 gene is reported to be transcribed last among the mast cell proteases during differentiation of both CTMC and MMC (9). We did not detect the expression of MMCP-4 mRNA in P815 cells after intraperitoneal culture (data not shown), suggesting that P815 cells did not differentiate to the stage at which MMCP-4 mRNA is expressed. The expression profiles of MC-CPA, MMCP-6, MMCP-2, and MMCP-4 mRNA, taken together, show that P815 cells maintained in the peritoneal cavity acquire the feature of partially rather than fully differentiated mast cells.

Various transcription factors act in combinations to regulate stage- and cell-type specific gene expressions during differentiation. The relationship between the function of transcription factors and their effects on cell differentiation has been studied extensively in erythroid lineage. Transcription factors involved in the differentiation of erythroid lineage such as GATA-1, GATA-2, and GATA-3 are also expressed in mast cells (17, 19). Moreover, GATA factors play important roles in the transactivation of mast cell specific genes. For example, MC-CPA gene is reported to be regulated by GATA factors in rodent mast cell lines (18). IL-4 gene is also trans-activated by GATA factors specifically in mast cells not in lymphocytes (19). We ourselves

Fig. 7. Regulation of transactivation by p45 and MafK through HDC 5'-flanking region. Quail fibroblast cell line QT-6 was transfected with pGL2-1099 and pEFp45 and/or pEFmMafK. The cells were harvested 48 h after transfection and assessed for the luciferase activity by a luminometer. The luciferase activity was normalized by dividing the count of the luciferase by β-galactosidase activity and expressed as the percentage of the value of control vector (pGL2-control vector). Each column was the averaged value ± S.E. of 4 experiments done in triplicate.
also observed that GATA-1 mRNA is expressed predominantly in peritoneal mast cells and MC/9 cells. Expression of GATA-1 mRNA was not detected in P815 cells in this study even after in vitro culture, suggesting that P815 cells did not differentiate fully after the in vitro culture and did not reach the stage for GATA-1 expression. This conclusion is consistent with that deduced from the MMCp mRNAs expression profiles (see above). On the other hand, GATA-2 mRNA was expressed at similar levels before and after intraperitoneal culture. Thus, GATA-2 may be the principle GATA factor that trans-activates MC-CPA gene.

Expression of p45 NF-E2 mRNA is largely restricted to the erythroid, megakaryocyte, and mast cell lineages (24). On the contrary, small Maf family proteins are found in a wide range of cell types, including erythroid, megakaryocyte, and mast cell lineages (24). On the other hand, small Maf family proteins are found in a wide range of cell types, including erythroid, megakaryocyte, and mast cell lineages (24).

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