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A Notch Ligand, Delta-Like 1 Functions As an Adhesion Molecule for Mast Cells

Akihiko Murata,* Kazuki Okuyama,* Seiji Sakano,† Masahiro Kajiki,‡ Tomohisa Hirata,‡ Hideo Yagita,‡ Juan Carlos Zúñiga-Pflücker,† Kensuke Miyake,‡ Sachiko Akashi-Takamura,‡ Sawako Moriwaki,‡ Shumpei Niida,† Miya Yoshino,* and Shin-Ichi Hayashi*

Mast cells (MCs) accumulate in chronic inflammatory sites; however, it is not clear which adhesion molecules are involved in this process. Recently, the expression of Notch ligands was reported to be upregulated in inflammatory sites. Although Notch receptors are known as signaling molecules that can activate integrins, their contributions to the adhesion of MCs have not been studied. In this study, we demonstrated that mouse MCs efficiently adhered to stromal cells forced to express a Notch ligand, Delta-like 1 (Dll1). Surprisingly, the adhesion was a consequence of direct cell–cell interaction between MCs and Dll1-expressing stromal cells rather than activation of downstream effectors of Notch receptor(s)-Dll1. The adhesion of MCs to Dll1-expressing stromal cells remained even when the cell metabolism was arrested. The recognition was blocked only by inhibition of Notch receptor(s)-Dll1 interaction by addition of soluble DLL1, or mAbs against Dll1 or Notch2. Taken together, these results indicate that Notch receptor(s) and Dll1 directly promote the adhesion of MCs to stromal cells by acting as adhesion molecules. This appreciation that Notch receptor–ligand interactions have an adhesion function will provide an important clue to molecular basis of accumulation of MCs to inflammatory sites. The Journal of Immunology, 2010, 185: 000–000.

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the recognition was blocked by addition of soluble DLL1, or mAbs against Dll1 or Notch2. Thus, our results show that Notch receptor (s) and Dll1 function as adhesion molecules for MCs.

Materials and Methods

Mice

C57BL/6J mice were purchased from Japan CLEA (Tokyo, Japan). Experiments were approved and performed in accordance with the guidelines of the Animal Care and Use Committee of Tottori University.

Reagents

Human IgG1 Fc-fused human DLL1 (DLL1-Fc), DLL4 (DLL4-Fc), JAG1 (JAG1-Fc), and Flag (FL)-tagged human JAG2 (JAG2-FL), which lacked the transmembrane and cytoplasmic domains were prepared as described (18, 19). Human IgG1 (Chemicon International, Temecula, CA) was used as a control for IgG1 Fc-fused Notch ligands. Sodium azide and DMSO were purchased from Wako Pure Chemical Industries (Osaka, Japan). A γ-secretase inhibitor, N-(3,5-difluorophenacetyl)-t-allyl]-S-phenylglycine ρ-buty l ester (DAPT) was purchased from Peptide Institute (Osaka, Japan).

Cell lines

OP9 stromal cell lines (20) carrying Dll1 and GFP genes (OP9-DL1), and GFP gene (OP9-control) were cultured in MEM (Toray Industries, Kanagawa, Japan) (Supplemental Table I) (30). The effective dose of DAPT was determined by adipocyte differentiation assay. OP9 cells differentiate into adipocytes, and Notch signaling inhibits adipocyte differentiation (31, 32). Forty-eight–well flat-bottomed culture plates (Corning Costar, Corning, NY) were coated with 1 μg DLL1-Fc, DLL4-Fc, JAG1-Fc, JAG2-FL, or human IgG1 in 100 μl PBS for 1 d at 4°C. After washing, OP9-control (2 × 10^3) in 200 μl of its culture media were seeded with or without DAPT or the same volume of its solvent, DMSO (0.1% v/v). After culturing for 5 d at 37°C, cells were fixed with 3.7% formaldehyde (Wako), and stained with Oil Red O (Sigma-Aldrich) solution. Stained cells were counted under a microscope (Fig. 3C, 3D).

Cell adhesion assay

OP9-control and OP9-DL1 (2 × 10^3) were seeded in 48-well plates and cultured for 2 d at 37°C to prepare confluent monolayers. After washing wells with PBS, MCs (1.5 × 10^5) suspended in 200 μl αMEM, supplemented with 10% FBS, were seeded into each well with or without MCs or reagents, and incubated for 1 h at 37°C in a humidified atmosphere of 5% CO2 in the air, unless otherwise indicated. Nonadherent MCs were recovered after vigorous agitation (low speed, scale 5) for 30 s with a Micro-Mixer E-36 (Taisei Corporation, Saitama, Japan), and were counted with a hemacytometer. Numbers of adherent MCs or ratios of nonadherent MCs relative to ones initially added in a well were calculated.

For the assay with fixed stromal cells, confluent monolayers of OP9-control or OP9-DL1 were fixed with 4% paraformaldehyde (Wako) for 5 min at room temperature. After washing cells, the adhesion assay was performed.

ST2NIC cells, a BM-derived stromal cell line (22) transfected with a constitutively active form of the Notch1 ICD gene, whose expression was controlled by tetracycline (Tc; Sigma-Aldrich, St Louis, MO)-off system and reflected by the expression of GFP, were maintained as described (22). Cells were cultured with RPMI 1640 (Life Technologies-BRL), supplemented with 5% FBS, 50 μM 2-ME (Wako) and 1 μg/ml Tc.

Results

MCs adhered to OP9-DL1 more efficiently than to OP9-control cells

We used OP9-DL1 as a model for tissues increased Dll1 density. Semiquantitative RT-PCR analysis showed that OP9-DL1 expressed more Dll1 than OP9-control, whereas Jag1 was expressed equally by both stromal cells (Fig. 1A). Surface Dll1 expression on OP9-DL1 was detected by flow cytometry (Fig. 1B). To assess whether Dll1 contributes to the adhesion of MCs to stromal cells, we incubated serial numbers of MCs on confluent monolayers of OP9-control or OP9-DL1 for 1 h. We then counted nonadherent MCs and calculated the numbers of adherent MCs relative to ones initially added in a well. The survival of MCs was not impaired during the adhesion assay (Supplemental Fig. 1). There were significantly more adherent MCs on OP9-DL1 than on OP9-control at each MC density (Fig. 1C). Numbers of adherent MCs on OP9-control cells reached a maximum when we incubated 8×10^5 MCs. In contrast, numbers of adherent MCs on OP9-DL1 continued to increase with MC density.

To assess the relationship between incubation time and adhesion efficiency, we incubated 1.5 × 10^5 MCs on OP9-control or OP9-DL1 for 15–240 min. The percentage of nonadherent MCs on OP9-DL1 was significantly lower than that on OP9-control at each time point (Fig. 1D). One hour was needed to reach a plateau of adhesion of MCs to OP9-control. In contrast, MCs stably adhered to OP9-DL1 within 15 min. These results suggest that Dll1 on stromal cells contributes to the efficient adhesion of MCs. Similar results were obtained from MCs induced from adult spleen or fetal liver (Fig. 4D).

The adhesion of MCs to OP9-DL1 was not inhibited by treatment with antagonistic Abs against Kit and integrin α5

A receptor protein tyrosine kinase, Kit, is one of the important adhesion molecules for MCs (33). MCs highly express cell surface
Kit molecules (Fig. 2A). Semiquantitative RT-PCR showed that both OP9-control and OP9-DL1 expressed comparable amounts of the Kitl, which consisted of membrane bound and soluble forms (Fig. 2B).

To investigate the contribution of Kit to the adhesion of MCs to stromal cells, we treated the cocultures with anti-Kit antagonistic mAb. Treatment with anti-Kit mAb was significantly effective on the adhesion of MCs to OP9-DL1 (Fig. 2C). These results indicate that Kit plays a critical role in the adhesion of MCs to OP9-DL1.

Notch signaling in stromal cells did not account for the efficient adhesion of MCs to OP9-DL1

RT-PCR analysis showed that MCs expressed Notch1 and Notch2, and both OP9-control and OP9-DL1 comparably expressed Notch1, Notch2, and Notch3 (Fig. 3A, Supplemental Table I), suggesting that the efficient adhesion of MCs to OP9-DL1 resulted from additional adhesion molecules on MCs or OP9-DL1 by Notch signaling. There are at least three possible explanations for the efficient adhesion: 1) OP9-DL1 cells expressed additional adhesion molecule(s) after interaction with MCs by reciprocal signaling via Notch between cells; 2) OP9-DL1 cells expressed additional adhesion molecule(s) by Notch signaling after interaction with each other; and 3) MCs expressed additional adhesion molecule(s) by signal transduction including Notch signaling after interaction with OP9-DL1.

To assess the first possibility that OP9-DL1 expressed additional adhesion molecule(s) by reciprocal signaling between MCs, we incubated MCs with fixed stromal cells. Fixed stromal cells would be unable to express additional cell surface molecules after interaction with MCs, though they would still provide cell surface molecules expressed before the adhesion assay. MCs also adhered to fixed OP9-DL1 more efficiently than to fixed OP9-control cells (Fig. 3B), indicating that the additional adhesion molecule(s) on OP9-DL1 after interaction with MCs did not account for the efficient adhesion of MCs to OP9-DL1.

To assess the second possibility that OP9-DL1 expressed additional adhesion molecule(s) following Notch signaling, we used a γ-secretase inhibitor, DAPT, which can block Notch cleavage at the transmembrane site and thus impairs Notch signaling (35). The effective dose of DAPT was determined by adipocyte differentiation assay (see Materials and Methods). Adipocyte differentiation of OP9-control was significantly inhibited by stimulation with immobilized Notch ligands but not control human IgG1 (Fig. 3C). Inhibition of adipocyte differentiation by immobilized DLL1-Fc was blocked by treatment with 10 μM but not 1 μM DAPT or DMSO during the culture (Fig. 3D). OP9-DL1 cells also had reduced tendency to differentiate into adipocytes and treatment with 10 μM DAPT was also effective on this inhibition (Supplemental Fig. 2). Therefore, we selected the 10 μM concentration of DAPT to inhibit Notch signaling.

If the efficient adhesion of MCs to OP9-DL1 resulted from the Notch signaling in stromal cells, pretreatment of stromal cells with DAPT would inhibit the adhesion of MCs. To this end, we pretreated OP9-control or OP9-DL1 with DAPT or DMSO for 2 to 3 d and then performed the adhesion assay. These reagents had no effect on the adhesion of MCs to either type of stromal cells (Fig. 3E).
If the efficient adhesion of MCs to OP9-DL1 arose from the Notch signaling in stromal cells, stimulation of Notch signaling in OP9-control might promote the adhesion of MCs. To this end, we incubated MCs on OP9-control stimulated with immobilized DLL1-Fc or human IgG1 for 2 d. These manipulations also had no effect on the adhesion of MCs (Fig. 3F). We assessed possible differences in expression of molecules associated with Notch signaling and cell adhesion between OP9-control and OP9-DL1 by cDNA microarray analysis. No significant difference except Dll1 was observed (Supplemental Table I). Flow cytometric analysis also showed that both stromal cells comparably expressed Vcam1, integrin αv, α5, and β1, and Cd44 (Supplemental Fig. 3).

To further investigate the importance of Notch signaling in stromal cells, we used ST2NIC cells carrying Notch1 ICD, a constitutive active form of Notch1 regulated under the Tc-off system (22). In the ST2NIC cells, the expression of Notch1 ICD was induced in the absence of Tc (Fig. 3G). We incubated MCs with ST2NIC cells precultured with serial doses of Tc for 3 d, and observed that the ratio of nonadherent MCs on ST2NIC cells was increased with the expression of Notch1 ICD (Fig. 3H, Supplemental Fig. 4). Taken together, these results indicate that Notch signaling in stromal cells did not account for the efficient adhesion of MCs to OP9-DL1.

Notch signaling in MCs did not account for the efficient adhesion of MCs to OP9-DL1

To assess the third possibility that MCs expressed additional adhesion molecules by Notch signaling after interaction with OP9-DL1, we treated the cocultures with DAPT or DMSO during the adhesion assay. These reagents had no effect on the adhesion of MCs to either OP9-control or OP9-DL1 (Fig. 4A).

To further assess the contribution of signal-transduction in MCs on their adhesion, we suppressed all possible expression of additional adhesion molecules during the adhesion assay. Cocultures were treated with sodium azide, which inhibits the function of ATPase and thus impairs ATP-dependent cell metabolism (36, 37). Treatment with sodium azide significantly inhibited the adhesion of MCs to OP9-control but not to OP9-DL1 (Fig. 4B). Moreover, simultaneous treatment with sodium azide and anti-Kit mAb completely inhibited the adhesion of MCs to OP9-control. Surprisingly, even in this condition, MCs were still adhered to OP9-DL1 cells and the percentage of nonadherent MCs was only 33.3 ± 3.9%.

In addition, we performed the adhesion assay on ice to arrest almost all signal-transduction and cell metabolism (37). It is known that Kit molecules on MCs are immediately internalized after binding to soluble Kit ligand (38). Because the expression of Kit on MCs is subject to a rapid turnover even when cells are at rest (38), their expression on MCs is rapidly recovered after treatment with trypsin. In cultures on ice, soluble Kit ligand-induced Kit internalization and recovery of Kit expression after treatment with trypsin on MCs were inhibited (data not shown). This indicates that turnover of cell surface protein is inhibited on ice. Conducting the adhesion assay on ice completely inhibited the adhesion of MCs to OP9-control, but more than 60% of MCs still adhered to OP9-DL1 (Fig. 4C). Similar results were obtained from MCs induced from adult spleen and fetal liver (Fig. 4D).

MCs were spherical and looked refractile by phase-contrast microscopy. Once tightly adhered to stromal cells, MCs spread on stromal cells and looked dark (Fig. 4E, upper). Interestingly, all of MCs still bound to OP9-DL1 in the presence of sodium azide and anti-Kit mAb or on ice were spherical and refractile (Fig. 4E, lower). This appearance may correspond to the tethering phase of cell adhesion (39).

These results suggest that the efficient adhesion of MCs to OP9-DL1 did not result from adhesion molecule(s) additionally expressed on MCs by any signal transduction after interaction with OP9-DL1. This means that adhesion molecule(s) that strongly support the adhesion of MCs must exist on the surface of OP9-DL1 stromal cells.

Notch receptor(s) and Dll1 themselves functioned as adhesion molecules for MCs

The previous findings begged the question of whether Dll1 could itself function as an adhesion molecule for MCs. To examine this,
we added soluble DLL1-Fc to the coculture as an antagonist. The adhesion of MCs to OP9-DL1 was inhibited by soluble DLL1-Fc but not human IgG1 in a dose-dependent manner (Fig. 5A). Addition of soluble DLL1-Fc at 30 μg/ml significantly inhibited the adhesion of MCs to OP9-DL1, and the percentage of nonadherent MCs on OP9-DL1 became comparable to that on OP9-control (25.1 ± 1.4% on OP9-control, 28.0 ± 1.0% on OP9-DL1 [p = 0.16]) (Fig. 5B, third columns). Moreover, almost all of the adhesion of MCs remained on OP9-DL1 held on ice was inhibited in the presence of soluble DLL1-Fc (Fig. 5C). Addition of anti-Dll1 mAb also significantly inhibited the adhesion of MCs to OP9-DL1 on ice (Fig. 5E). These findings indicate that Dll1 on OP9-DL1 functions as an adhesion molecule, directing the remarkable adhesion of MCs to OP9-DL1.

We also assessed whether the Dll1-associated adhesion was inhibited by other soluble Notch ligands. The adhesion of MCs to OP9-DL1 was also inhibited by soluble DLL4-Fc (Fig. 5B). Interestingly, soluble JAG ligands did not inhibit the adhesion of MCs to OP9-DL1. None of soluble Notch ligands tested here inhibited the adhesion of MCs to OP9-control (Fig. 5B).

Dll1 was reported to interact with Notch1 and Notch2 (40, 41), and MCs expressed both receptors (Fig. 5D, Supplemental Fig. 5A) (42). To assess whether Notch receptors are counter-receptors for Dll1, we treated the coculture with anti-Notch2 mAb (26). The...
adhesion of MCs to OP9-DL1 on ice was significantly inhibited by anti-Notch2 mAb at a concentration of 100 µg/ml in nine of 10 experiments (Fig. 5E). The effect of simultaneous addition of anti-Dll1 and anti-Notch2 mAbs was comparable to that of single applications of them (Fig. 5E). These results indicate that at least Notch2 on MCs functions as an adhesion receptor and permits binding to Dll1.

Taken together, Notch receptor(s) and Dll1 themselves function as adhesion molecules and contribute to the efficient adhesion of MCs to stromal cells.

Discussion

Our findings describe a new mechanism through which Notch family members can mediate cell–cell communication. A Notch ligand, Dll1 effectively functions as an adhesion ligand, binding MCs to stromal cells. This study was initiated because of reports that Notch ligand levels are elevated in inflammatory sites where MCs accumulate, and our observations provide an important insight into molecular basis of cell accumulation.

The adhesion to OP9-DL1 occurred even when cell metabolism was arrested and did not require induced expression of additional molecules. Importantly, it was inhibited by addition of soluble Dll1 ligands. Several studies have suggested that soluble Notch ligands can activate Notch signaling in some conditions (43–45); however, it is not the case in this study because Notch signaling was irrelevant to the adhesion of MCs to OP9-DL1.

Members of the Notch family are potential counter-receptors for Dll1. Addition of anti-Notch2 mAb significantly inhibited the adhesion of metabolically inactive MCs to OP9-DL1, indicating that Notch2-Dll1 interactions have an adhesion function. Remaining MC adhesion to OP9-DL1 in the presence of anti-Notch2 mAb was significantly inhibited by further addition of recombinant mouse Notch1-Fc as an antagonist (Supplemental Fig. 5), implying that Notch1–Dll1 interaction might also contribute to the adhesion of MCs.

We assessed whether the Jag ligand could also contribute to the adhesion of MCs using JAG2-expressing fibroblasts as a substitute for OP9-DL1, and observed that MCs also adhered to them more efficiently than to control fibroblasts (Supplemental Fig. 6). This efficient adhesion of MCs was not inhibited by addition of anti-Kit mAb, but inhibited by culturing on ice (Supplemental Fig. 6). These observations support that Jag2 also promotes the adhesion of MCs, although it might not directly function as an adhesion ligand.

In studies of adhesion molecules, the affinity characterized by a $K_d$ is an important index of adhesion force. We previously observed that $^{125}$I-labeled IgG1 Fc-tagged soluble DLL1 bound to CCRF-CEM T cell line expressing Notch1, Notch2, and Notch3 with an apparent $K_d$ of 9.23 nM (Supplemental Fig. 7). The $K_d$ of soluble Jag1 for Ba/F3 cells was reported to be 0.4 nM (46). Previous studies showed the $K_d$ of other adhesion receptor–ligand pairs involved in cell–cell interactions; soluble P-selectin for neutrophils was $\sim$70 nM (47); soluble Icam1 for Lfa1 on activated T cells was 400 nM (48); soluble VCAM1 for $\alpha$4$\beta$1 integrin on U937 cells was 33 nM (49). These results indicate that the affinity of Notch receptor–ligand interactions is relatively high compared with those of other adhesion receptor–ligand pairs. Analysis of purified or recombinant receptor–ligand protein binding also showed comparable results (Supplemental Table II). This high affinity might allow Notch receptor–Dll1 interactions to function in cell adhesion.

Interestingly, soluble JAG ligands did not inhibit the adhesion of MCs to OP9-DL1 contrary to soluble DLL ligands at the same concentration. This result suggests that the affinity of Dll ligands to Notch receptors on MCs is higher than that of Jag ligands. The glycosylation of Notch receptors by fringes is known to influence the Notch receptor–ligand binding specificity. Fringes are fucose-specific $\beta$1,3-N-acetylgalactosaminyltransferases localized to the Golgi, which elongate O-linked fucose residues on Notch ECD (50, 51). Several reports have suggested that fringe modification enhances the affinity of Dll but not Jag ligands to Notch receptors (40, 41, 52, 53). Because MCs expressed three homologs, Lunatic, Manic, and Radical fringes (RT-PCR analysis, data not shown), Notch receptors on MCs might be potentiated to bind soluble Dll ligands selectively.

The expression of Notch receptors is widely detected through hematopoietic cell lineages (14, 15). We observed that T cells and B cells in lymph nodes of naive C57BL/6 mice also adhered to OP9-DL1 more efficiently than to OP9-control, and their adhesion to OP9-DL1 still remained in cultures on ice (A. Murata, unpublished data). Unlike MCs, however, their recognitions were not inhibited by addition of soluble DLL1 (30 µg/ml) or anti-Notch2 mAb (100 µg/ml) (A. Murata, unpublished data). These results suggest that the mechanism that Dll1 promotes cell adhesion might be different between MCs and lymphocytes.

Despite their expression of both Notch receptors and Dll1, OP9-DL1 cells do not tend to form cell aggregation or adhere tightly each other. Notch ligands are known to form cis interactions with Notch receptors expressed in the same cell (54, 55). The cis interaction is shown to inhibit the trans-activation of Notch receptors by Notch ligands expressed on adjacent cells (55–57). It might mean that the cis interactions inhibit the trans interactions of Notch receptors and Dll1, resulting in abrogation of the self-adhesion of OP9-DL1.

The adhesion function of Notch receptors requires intact Notch ECD, whereas the signaling function requires proteolytic cleavage of Notch ECD by ADAMs (14). This is interesting because these functions of Notch receptor–ligand interaction are exclusive and irreversible. ADAMs might not only initiate Notch signaling but also diminish cell adhesion mediated by Notch receptor–ligand interactions. To determine how the function of ADAMs is regulated will provide an insight of how these two functions of Notch receptor–ligand interactions are controlled.

Homologs of Notch receptors and their ligands have been identified in a variety of multicellular organism (58). It is noteworthy that Drosophila Notch–Delta interaction is reported to have a high adhesion force (59). When Drosophila Schneider (S2) cells expressing Notch are mixed with S2 cells expressing Delta, huge cell aggregates are formed (60). Overexpressed zebrafish Delta in cultured human keratinocytes also promotes cell cohesiveness (61). Moreover, Ba/F3 cells, which hardly adhere to the Chinese hamster ovary cell line, can adhere to that expressing mouse Dll1 (62). These results are not only consistent with our finding that Dll1 functions as an adhesion molecule but also suggest that its adhesion function might be evolutionally conserved.

It is still vague for what kinds of physiological situation Notch receptor–ligand interactions play important roles through MCs. Our observations suggest that Notch receptors and their ligands might be involved in recruitment and retention of MCs in tissues as adhesion molecules. Recently, Notch2 signaling is reported to induce differentiation of MCs from BM progenitor cells in vitro, but MCs were not depleted in Notch2 conditional knockout mice (63). The expression level of Notch ligands is reported to be up-regulated at sites of chronic inflammatory diseases (8–10, 64–66), where are frequently accompanied by the accumulation of MCs (4, 11, 12). These results suggest that Notch receptor–ligand interactions might be important for accumulation of MCs in inflammatory but not normal condition. Notch ligands are upregulated on endothelial cells in inflammatory sites (64, 67, 68), suggesting that they might contribute to the process of cellular extravasation. In
fact, MCs were tethered to OP9-DL1 in cultures held on ice only by Notch receptor(s)–DII interactions. In addition, Notch signaling is reported to confer APC function on MCs by inducing the expression of MHC class II and OX40 ligand (42). In view of these evidences, Notch receptor–ligand interactions could affect the process of inflammation through modulating cell adhesion, differentiation, and effector functions of MCs.

Notch receptor–ligand interactions are critical to a wide range of biological processes that range from normal development to malignancy. This appreciation that in addition to signaling and activation of transcription, cell adhesion is involved provides a new perspective on these issues. Determining the relationship between adhesion and signaling functions of Notch receptors and their ligands should dissect these important processes.

Acknowledgments

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Disclosures

S.S., M.K., and T.H. are employed by Asahi Kasei Corp. The other authors have no financial conflicts of interest.

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8 CELL ADHESION BY A NOTCH LIGAND


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