N-Cadherin Plays a Role in the Synapse-Like Structures between Mast Cells and Neurites

Akio SUZUKI, a Ryo SUZUKI, a Tadahide FURUNO, a Reiko TESHIMA, b and Mamoru NAKANISHI* a

a Graduate School of Pharmaceutical Sciences, Nagoya City University; Tanabe-dori, Mizuho-ku, Nagoya 467–8603, Japan; and b National Institute of Health Science; Kamiyoga, Setagaya-ku, Tokyo 158–8501, Japan.

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Communication between nerves and mast cells is a prototypic demonstration of neuro-immune interaction. Numerous studies have shown that the stimulation of nerves (or addition of neurotransmitters) can evoke activation of mast cells, and that mast cell-derived mediators can influence neuronal activity. However, the molecules involved in the membrane-membrane contacts between nerves and mast cells are still unknown. Here, we used an in vitro co-culture approach comprising interaction between immune (bone marrow-derived mast cell, BMMC) and nerve cells (superior cervical ganglia, SCG). The experiments showed clearly that the nerve-mast cell communication was supported by synapse-like structure and that N-cadherin, not E-cadherin, played an essential role in the synapse-like structure. In addition, we found that the synapse-like structure was assisted by clustering of β-catenin to N-cadherin.

Key words  neuro-immune interaction; mast cell; N-cadherin; confocal laser scanning microscopy; superior cervical ganglia; β-catenin

During the last decade there has been an exponential increase in data illustrating that the nervous and immune systems are not disparate entities. 1–2 The nerve-mast cell relationship has served as a prototypic association and has provided substantial evidence for bi-directional communication between nerves and immune cells. 3 Early studies elegantly described the non-random spatial association of nerves and mast cells in a variety of tissues in which actual membrane-membrane contacts could be observed. 4–5 To understand these events we have studied recently direct neurite-mast cell (RBL) communication using an in vitro co-culture approach and a calcium imaging by confocal laser scanning microscopy (CLSM). Our results showed clearly that nerve-mast cell communication can occur in the absence of an intermediary transducing cell and that the neuropeptide substance P, operating via NK-1 receptors, is an important mediator of this communication. 6 Our findings have implications for the neuroimmune signaling cascade that are likely to occur during airways inflammation, intestinal hypersensitivity, and other conditions in which mast cells feature. 6–9 In addition we studied by atomic force microscopy (AFM) the morphological structure of the interaction between neurites and mast cells (RBL) which was involved in the communication. 9 AFM images showed that association between the growth cone of neurites and the RBL cell occurred over ca. 7 μm and that direct neurite-mast cell cross-talk induced degranulation in RBL cells via the neuropeptide substance P.

In the present paper we have focused on the molecular mechanism of the membrane proteins involved in the synapse-like structure between neurites and mast cells. We used an in vitro co-culture approach comprising superior cervical ganglia (SCG) and mouse bone marrow-derived mast cells (BMMC) besides RBL cells. Mast cells are classified into two types. One is mucosal and another is connective tissue type. Both RBL cells and BMMCs belong to mucosal type mast cells. However, RBL cells are much more adhesive than BMMCs and would like to non-specifically stick to the supported matrigel-coated plate of glass dishes. They also have a malignant feature. In addition, nerves most commonly associated with mast cells in vivo contain substance P. 7 Sub-stance P in isolated neurons from SCG maintained in culture showed a 25-fold increase within 48 h in vitro. 10 Therefore, we studied an in vitro co-culture approach comprising SCG and BMMC to understand precisely adhesion proteins that play a role in the neurite-mast cell communication.

Cadherins are a family of calcium-dependent and homophilic cell adhesion proteins that play in the formation of synaptic plasticity. E- and N-cadherins, which are classic cadherins, are found in synapses and they appear to straddle the presynaptic and postsynaptic membranes surrounding the active zone and the postsynaptic density. 11 Cadherins mediate strong adhesion through intracellular interaction with β-catenin, which in turn associates with α-catenin and the actin cytoskeleton. 12 E- and N-cadherins were expressed on bone marrow-derived cells as well as on epithelial cells or neuronal cells. It was shown that E- and N-cadherins, α- and β-catenins, and p120 catenin were expressed in BMMC, and that E-cadherin and associated cytoplasmic proteins might be involved in the regulation of certain stage of mast cell differentiation and cell-cell interactions. 13 However, it was unclear until now whether cadherins and/or associated proteins were concerned in the contacts between neurites and mast cells. The present experiments indicated that both N-cadherin and E-cadherin in BMMC were spread over in the cytoplasm of BMMC without association of the neurites. With association of the neurites, however, N-cadherin was localized on the plasma membranes of BMMCs but E-cadherin was not. Such kind of localization was also observed for β-catenin which was supposed to assist localization of N-cadherin on the plasma membranes. This provides a new insight into the molecular mechanism in neuro-immune communication between neurites and mast cells.

* To whom correspondence should be addressed. e-mail: mamoru@phar.nagoya-cu.ac.jp © 2004 Pharmaceutical Society of Japan
MATERIALS AND METHODS

Materials  Bradykinin was purchased from Bachem (Bubendorf, Switzerland). Fluo 3-AM was obtained from Dojindo (Kumamoto, Japan). Mouse anti-N-cadherin and mouse anti-E-cadherin antibodies were obtained from Becton Dickinson (San Jose, CA, U.S.A.), and rabbit anti-β-catenin antibody was from Sigma (St. Louis, MO, U.S.A.). CP-99994-01, an NK-1 receptor antagonist, was from Pfizer (Groton, CT, U.S.A.).

Mast Cell Culture  BMMCs were obtained by culturing bone marrow cells from 10—15-week-old female BALB/c mice (Japan SLC, Shizuoka, Japan) in DMEM supplemented with 10% fetal calf serum (FCS) (Roche, Mannheim, Germany), 0.1 mM non-essential amino acids (Invitrogen, Carlsbad, CA, U.S.A.), 100 U/ml penicillin, 100 µg/ml streptomycin, and 10 ng/ml recombinant mouse IL-3 (Genzyme Tech, Minneapolis, MN, U.S.A.). RBL cells were cultured according to previously described procedure.6)

Nerve-Mast Cell Co-culture  Following a published protocol,14,15) SCG were dissected from newborn (<48 h old) CBA mice (Japan SLC, Shizuoka, Japan). The neurons were plated on matrigel-coated 35-mm diameter glass dishes (Matsunami, Japan) and maintained in F-12 medium (Invitrogen) supplemented with 0.2 mM L-glutamine, 0.3% glucose, 3% antibiotic/antimycotic, 10% FCS, and 50 ng/ml nerve growth factor (NGF) (Upstate Cell Signaling Solution, 3% antibiotic/antimycotic, 10% FCS, and 50 ng/ml recombinant mouse IL-3 (Genzyme). RBL cells were co-cultured with SCG according to previously described procedure.6)

Scanning Electron Microscopy (SEM) and Confocal Laser Scanning Microscopy (CLSM)  For SEM studies, cells were fixed for 2 h with 2% glutaraldehyde in 200 mM Sörensen’s phosphate buffer solution (pH 7.4) and were dried in the air. They were coated with platinum and observed under a scanning electron microscope (S-4300; Hitachi, Tokyo, Japan). CLSM was done using a confocal laser scanning microscope (LSM-510; Zeiss, Oberkochen, Germany).5) Fluo-3 and FITC fluorences were observed using excitation and emission wavelengths of 488 nm and >505 nm, respectively.

Cellular Activation  After co-culture for 3 d, cells were treated for 30 min with culture medium containing 1 µM Fluo 3-AM at 37°C, and then washed twice with Hepes buffer (10 mM Hepes (pH 7.2), 140 mM NaCl, 5 mM KCl, 0.6 mM MgCl2, and 1 mM CaCl2, 0.1% bovine serum albumin, 0.1% glucose, and 0.01% sodium pyruvate). Neurite activation was evoked by 10 nM bradykinin at 37°C under CLSM. Experimental results are expressed as mean and SEM. Results were tested statistically by unpaired two-tailed Student’s t test. Results were considered statistically significant when p<0.01.

Immunostaining  BMMCs were plated on poly-L-lysine-coated glass chamber (Elecon, Chiba, Japan). SCG and BMMCs were co-cultured on matrigel-coated glass dishes. Cells were fixed by 3% paraformaldehyde for 15 min and were permeabilized by 0.2% Triton X-100 for 5 min. After blocking with 10% BSA, cells were probed with anti-N-cadherin (1:125 dilution), anti-E-cadherin (1:125 dilution), or anti-β-catenin (1:500 dilution) antibodies and treated with FITC-conjugated goat anti-mouse IgG (1:125 dilution; Cappel, Aurora, OH, U.S.A.) or FITC-conjugated goat anti-rabbit IgG (1:500 dilution; Santa Cruz Biotechnology, Santa Cruz, CA, U.S.A.). Samples of immunostaining were observed by CLSM.

RESULTS

After 3 d co-culture of neurites with RBL cells or BMMCs the membrane-membrane contacts were formed on the matrigel-coated glass dish as shown in Fig. 1. To study the morphological interaction between neurites (SCG) and mast cells (RBL cells or BMMCs) we observed their contacts on the cover glass by SEM. Electron microscopic images of coculturing SCG and RBL showed that they formed the synapse-like structure as shown in Figs. 1a and b. Figure 1a is a typical example of SCG-RBL contacts and Fig. 1b is an enlarged one of Fig. 1a. A terminal of the thin SCG neurites extended and attached to the cell body of an RBL cell. In the case of BMMCs, however, the neurite-mast cell contacts were quite different as shown in Fig. 1c. The interaction was not so tight and BMMCs were usually attached to neurites along their thin fiber (see Fig. 1c). The interaction between BMMCs and neurites was supported by gathering several thin neurites along a BMMC as shown in Fig. 2. Figure 2 was observed by confocal laser scanning microscopy (CLSM). Figure 2a is a representative differential interference contrast (DIC) image of the neurite-BMMC co-culture system. We loaded calcium fluorophore Fluo-3 to the SCG-BMMC co-culture system in Fig. 2b. Addition of bradykinin to co-cultures resulted in an increase of Ca2+ concentration in

Fig. 1. Electron Microscopic Images of Coculturing SCG and RBL (or BMMC)
(a) A typical example of SCG-RBL contacts. A white bar is 20 µm. (b) An enlarged image of Fig. 1a. A white bar is 5 µm. (c) A typical example of SCG-BMMC contacts. A white bar is 10 µm.
neurites within 5 s (Fig. 2c). This neurite activation event was followed by BMMC activation, indicated by a Ca\(^{2+}\)/H11001 increase in the BMMCs in contact with activated neurites (Fig. 2d). It was confirmed that spherical cells on neurites (white arrows in Fig. 2a) were BMMCs. They had Fc receptor and were activated by the binding of the specific antigen.

A typical example of the time-course of Fluo-3 fluorescence intensity changes is shown in Fig. 3a. Pretreatment of neurite-BMMC co-cultures with 100 ng/ml CP-99994-01, an NK-1 receptor antagonist, for 30 min inhibited significantly a Ca\(^{2+}\)/H11001 increase in BMMCs subsequent to neurite activation by bradykinin stimulation as shown in Fig. 3b. The percentages of responding BMMCs to neurite activation were 53.1 ± 3.5% and 25.8 ± 6.6% without and with the pretreatment of CP-99994-01, respectively. The results were well consistent with the previous results for RBL cells. Bradykinin induced directly an increase of Ca\(^{2+}\) concentration in SCG neurites, but not in BMMCs. This indicated that BMMCs, like RBL cells, were activated via NK-1 receptors with substance P released from activated neurites.

In order to identify adhesion proteins involved in this functional communication between the neurites and mast cells, we studied the expression of N- and E-cadherins in both SCG and BMMCs. Western blotting analysis showed that N- and E-cadherins were expressed both in SCG and in BMMCs (data not shown).

Further, we also studied the changes of localization of cadherins in BMMCs in the co-culture. N-cadherin was distributed in the cytoplasm of BMMCs in the absence of SCG neurites (Fig. 4, Ia and Ib). In Fig. 4, Ib, N-cadherin was distributed in the left side of the cytoplasm. Non-fluorescent area in the right side was due to the nucleus of a BMMC. In this case the fluorescence on the plasma membrane was very weak. After 3 d in co-culture, N-cadherin would like to localize on the plasma membrane of the BMMC which was attached with the neurites in Fig. 4, Ic and Id. N-cadherin molecules of BMMC were significantly gathered along the contact region with neurites although weak but apparent fluorescence in the thin fiber of neurites was observed (Fig. 4, Id). Therefore, homophilic association seemed to be occurred between BMMCs and SCG neurites via N-cadherin. On the other hand, E-cadherin was also distributed in the cytoplasm of BMMCs in the absence of neurites (see the fluorescence of the left side of the BMMC in Fig. 4, Ila and IIb), but it did not change the localization in BMMCs with association of neurites (Fig. 4, IIc and IId). In addition, β-catenin which is known to be an associated protein with cadherins, was distributed in the cytoplasm without neurites (see the fluorescence of the lower part of the BMMC in Fig. 4, IIIa and IIIb). It was accumulated from the cytoplasm to the periphery of the plasma membrane in the BMMC which was attached to the neurites (Fig. 4, IIId and IIIId). These results indicated that N-cadherin, but not E-cadherin, was involved with the contact formation between nerves and mast cells in collaboration with β-catenin.

**DISCUSSION**

Nerve-mast cell associations have been reported within peripheral, myelinated nerves, unmyelinated nerves, neurofibroma, and neuroma. A morphological study showed that mast cells and nerves were closely and invariably approxi-
mated in rat intestinal villi. Electron microscopy showed apparent membrane-membrane association between mucosal mast cells and nerves with dense core vesicles at the points of contact. The nerves in contact with mast cells contained either substance P, CGRP, or both. Similar observations in a variety of different tissues in many species suggest the involvement of mast cells in the neurogenic component of inflammatory conditions. Substance P can induce the release of histamine from peritoneal and mucosal mast cells, and human mast cells further supporting the functional interaction between mast cells and substance P-containing nerves. Using an in vitro co-culture system of SCG-RBL cells as a model of neuron-mast cell association, we previously found that the RBL cell activation was elicited as a direct consequence of contact with a specific activated nerve fiber and that this RBL activation was mediated largely via substance P release and through NK-1 receptors.

In present study, we have examined adhesion proteins concerned with functional interactions between nerves and mast cells using the co-culture system of SCG and BMMCs which are homologues of mucosal mast cells. We have shown that neurite activation could elicit Ca2+ mobilization in a coculturing BMMC and that the mobilization was inhibited by NK-1 receptor blockade. The result suggested the potential for neurons to direct BMMC activity in the absence of intermediary cells, as well as RBL cells.

Cell recognition and adhesion in many cell types is mediated by cadherins which comprise a superfamily of single-transmembrane, calcium-dependent, cell adhesion proteins. Cadherins associate with the cytoskeleton through cytoplasmic interactions with catenins. When we checked the cadherin expression in BMMCs and SCG, both of them expressed E- and N-cadherins. Immunostaining analysis showed that E- and N-cadherins resided mainly in the cytoplasm of BMMC. We have shown here that adhesion with neurites led to the accumulation of N-cadherin, but not E-cadherin, on the plasma membrane of BMMCs. Further, the accumulated N-cadherin on the plasma membrane in BMMCs was concentrated along the contact region with neurites. These results suggest that N-cadherin rather than E-cadherin is intimately concerned with the communication between neurites and BMMCs.

In neurons, the cadherin-catenin complex is well situated to facilitate the formation and maintenance of synaptic junctions, coordinate presynaptic and postsynaptic structural changes, and modulate the adhesiveness of synaptic junctions. Synaptic activity has been reported to modify the conformational state of N-cadherin. Impairment of cadherin adhesion blocks the early or late phase of long-term potentiation (LTP), and N-cadherin levels are significantly elevated during late LTP. Our findings support an important role of N-cadherin in nerve-mast cell interaction as well as in the nerve-nerve interaction.

Not only the nervous system but also the immune system utilizes synapse to directly convey and transduce highly controlled secretory signals between their constituent cell populations. The prototypic criteria for synapse have been developed by Dustin and Colman. At the electron microscopic level, it was shown that noradrenergic nerve terminal forms intimate contact with the surface membrane of T lymphocytes in spleen. The foregoing experiments may indicate an evidence for the biological and physiological functions of N-cadherin in neuro-immune communication, but clearly such localization of N-cadherin must be important in many conditions which nerves and mast cells have been implicated, including bronchial hypersensitivity, asthma, food allergy, and inflammatory bowel disease.

REFERENCES