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Title page

Increased expression of cell adhesion molecule 1 by mast cells as a cause of enhanced nerve–mast cell interaction in a hapten-induced mouse model of atopic dermatitis

M. Hagiwara*, T. Inoue*, T. Furuno[†], T. Iino[‡], S. Itami*, M. Nakanishi[†], H. Asada[§],
Y. Hosokawa[‡] and A. Ito*

* Department of Pathology, Faculty of Medicine, Kinki University, Osaka-Sayama, Osaka 589-8511, Japan

[†] School of Pharmacy, Aichi Gakuin University, Nagoya, Aichi 464-8650, Japan

[‡] Graduate School of Materials Science, Nara Institute of Science and Technology, Ikoma, Nara 630-0192, Japan

[§] Department of Dermatology, Nara Medical University, Kashihara, Nara 634-8522, Japan

Running head

Neuroimmunological role of mast cells in atopic dermatitis

Correspondence

Akihiko Ito, Department of Pathology, Faculty of Medicine, Kinki University, 377-2 Ohno-Higashi, Osaka-Sayama, Osaka 589-8511, Japan.

Telephone: +81 72 366 0221 ext.3140; Fax: +81 72 360 2028

E-mail address: aito@med.kindai.ac.jp

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Conflicts of interest

None declared.

What's already known about this topic?

- Enhanced sensory nerve–skin mast cell interaction is involved in the pathogenesis of atopic dermatitis (AD).

What does this study add?

- In a hapten-induced AD mouse model, lesional mast cells were found to express much higher levels of cell adhesion molecule-1 (CADM1) than non-lesional mast cells.
- CADM1 on mast cells enhanced sensory nerve–mast cell adhesion and communication *in vitro* in a dose-dependent manner.
- Increased CADM1 expression in mast cells appears to play a role in the pathogenesis of AD.

Summary

Background. Neuroimmunological disorders are involved in the pathogenesis of atopic dermatitis (AD), partly through enhanced sensory nerve–skin mast cell interaction. Cell adhesion molecule 1 (CADM1) is a mast-cell adhesion molecule that mediates the adhesion to, and communication with sympathetic nerves.

Objectives. To investigate the role of mast cell CADM1 in the pathogenesis of AD, CADM1 expression levels were compared between lesional and non-lesional skin mast cells of an AD mouse model, which was developed by repeated application of trinitrochlorobenzene.

Additionally, how the detected CADM1 alterations in lesional mast cells might affect the sensory nerve–mast cell interaction was examined in cocultures.

Methods. AD-like lesional and non-lesional skin mast cells were separately collected by laser capture microdissection. CADM1 expression was examined by reverse transcription-PCR and CADM1 immunohistochemistry. In cocultures adhesion between dorsal root ganglion (DRG) neurites and IC2 mast cells was analysed by loading a femtosecond laser-induced impulsive force on neurite-attendant IC2 cells, while cellular communication was monitored as the IC2 cellular response ($[Ca^{2+}]_i$ increase) after nerve-specific stimulant-induced DRG activation.

Results. AD-like lesional mast cells expressed three-fold more CADM1 transcripts than non-lesional cells. This was supported at the protein level, shown by immunohistochemistry. In coculture, CADM1 overexpression in IC2 cells strengthened DRG neurite–IC2 cell adhesion and doubled the population of IC2 cells responding to DRG activation. A function-blocking anti-CADM1 antibody abolished these effects in a dose-dependent manner.

Conclusions. Increased expression of CADM1 in mast cells appeared to be a cause of enhanced sensory nerve–mast cell interaction in a hapten-induced mouse model of AD.

Text

Introduction

Atopic dermatitis (AD) is a chronic and relapsing inflammatory skin disease characterised by eczematous skin lesions, xerosis, lichenification, and severe pruritus. While AD is assumed to have a multifactorial pathogenesis involving both genetic and environmental factors, previous clinical and laboratory studies indicated that neuroimmunological disorders are involved in the onset and exacerbation of AD.^{1,2} This theory is supported by the anatomical evidence that the number of mast cells and their contact with sensory nerve fibres are increased in AD lesional skin.³⁻⁵ Sensory nerve–mast cell contacts are proposed to be responsible for severe pruritus in AD, because substance P secreted from sensory nerve fibres induces the release of

various mediators from mast cells. These provoke both afferent excitation of histamine-sensitive C-fibres and neurogenic inflammation.^{6, 7}

To test the aetiology and pathophysiology of AD, a variety of animal models have been developed. Among the most well-known is a hapten-induced mouse model. In this, BALB/c or NC/Nga mice are sensitised to a hapten, such as trinitrochlorobenzene (TNCB), and are then repeatedly challenged with the same hapten. Within a month of initiation of the challenge, the mice develop AD-like skin lesions associated with an increased number of mast cells.^{8, 9} As in AD patients, this model mouse often results in scratching of the AD-like skin lesions, as a result of the activation of skin mast cells.¹⁰⁻¹²

In 2003, we identified a new mast-cell adhesion molecule, formerly called spermatogenic Ig superfamily (SgIGSF), and in 2007 renamed it cell adhesion molecule 1 (CADM1).¹³ Structurally, CADM1 is a membrane-spanning glycoprotein composed of three extracellular Ig-like domains, a single transmembrane region, and a short carboxy-terminal intracellular tail with a protein 4.1 interaction sequence and a PDZ type II motif.¹⁴ We previously cocultured murine mast cells and sympathetic superior cervical ganglion (SCG) neurons, showing that CADM1 mediated the adhesion of mast cells to SCG neurites and promoted SCG neurite-to-mast cell communication.^{15, 16}

In the present study, we compared the expression levels of CADM1 in mast cells from inflamed and non-inflamed ear auricles of a TNCB-induced mouse model of AD. We found that AD-like lesional mast cells expressed much higher levels of CADM1. To investigate whether this increased expression of CADM1 might explain enhanced nerve-mast cell interaction in AD, we cocultured murine mast cells and sensory dorsal root ganglion (DRG) neurons. The cocultures were analysed using two assays, one based on loading of a

femtosecond laser-induced impulsive force the other using imaging of a Ca^{2+} indicator fluorescence, both previously established in our laboratory.¹⁵⁻¹⁷

Materials and methods

Cells and antibodies

Bone marrow-derived cultured mast cells (BMMCs) from BALB/c mice were cultured as described previously.¹³ IC2 cells are an interleukin-3-dependent mast cell line derived from BMMCs of DBA/2 mice,¹⁸ and lack endogenous CADM1.¹⁵ They were maintained in our laboratory, and were modified to express CADM1 exogenously (IC2^{CADM1}), as described previously.¹⁶ Rabbit polyclonal and chicken monoclonal (9D2) antibodies were previously generated against the mouse CADM1 C-terminal oligopeptide and recombinant extracellular domain, respectively.^{13, 15} 9D2 is a function-blocking antibody to CADM1.¹⁵ A mouse monoclonal anti- β -actin antibody and control chicken IgY (U04) were purchased from Sigma-Aldrich (St. Louis, MO, U.S.A.) and R&D Systems (Minneapolis, MN, U.S.A.).

Animal model

The hapten-induced mouse model of AD was developed by Biostir Inc. (Kobe, Japan), according to a well-established protocol.⁸ Briefly, the abdomen of BALB/c mice (n = 10) was shaved and sensitised epicutaneously by a single application of 150 μl 1% TNCB solved in acetone. One week after sensitisation, the dorsal surface of the right ear auricle was challenged with 20 μl of the same TNCB solution. After 24 h, the ear auricles were removed from five mice. The right ears of the remaining five mice were repeatedly challenged with 1% TNCB every 2 days for 3 weeks. The left ears were left untreated over this time period. Both ear auricles were removed from the 5 mice.

Histological examination

The excised ear auricles were cut into two pieces; one was formalin-fixed and paraffin-embedded, the other was freshly frozen in aqueous mountant. Paraffin-embedded tissue sections were stained with haematoxylin and eosin or alcian blue and nuclear fast red using standard procedures. The number of alcian blue-stained cells present in the dermis (between the epithelial basement membrane and the ear cartilage) was counted throughout a 1-cm-long section of each auricle, while the dermal area was measured using morphometric software (DP72, Olympus, Tokyo, Japan). The number of alcian blue-stained cells present in a 1-mm² dermal area was calculated for each ear, and expressed as the number of mast cells/mm² [mean±standard deviation (SD)]. For immunohistochemistry, paraffin sections were reacted with a rabbit polyclonal anti-CADM1 antibody, as described previously.¹⁹ Immunoreactive signals were coloured with diaminobenzidine. Sections were then stained with alcian blue and nuclear fast red to visualise mast cells.

Laser capture microdissection (LCM)

Frozen ear blocks were cut into 6-µm-thick sections. Three serial sections were mounted onto a membrane-coated slide (Leica Microsystems, Wetzlar, Germany), and ten slides for each ear were prepared. Sections were fixed with ice-cold ethanol/acetate (19:1 vol/vol) for 3 min, washed in ice-cold RNase-free water to remove aqueous mountant, and stained with toluidine blue for 10 s. After washing twice with ice-cold RNase-free water for 1 min, the sections were cold air-dried in 2 min with a hairdryer, and were stored at -80°C until use. For LCM, the slides were quickly cold air-dried at a room temperature below 8°C, and were mounted upside down onto the frame support of a LMD7000 (Leica). Mast cells displaying metachromasia were individually excised from the sections and transferred into a 200-µl PCR tube containing Tris(2-carboxyethyl) phosphine hydrochloride. The collection time for one slide was limited to 30 min. Each cell pool was frozen in dry ice immediately after collection.

Reverse transcription (RT)-PCR followed by Southern blotting

Ten cell pools from one ear were combined into one tube immediately after thawing. Total RNA was extracted from the LCM-harvested cells using NucleoSpin RNA XS columns (Macherey-Nagel, Düren, Germany), according to the manufacturer's instructions. The procedures for RT-PCR analyses and details of the primers used were described previously.²⁰ For Southern blot analyses, PCR products were electrophoresed on 2% agarose gels and transferred to a nylon membrane using standard methods. For probe preparation, the PCR products from BMMCs' RNA were excised from the agarose gels. The subsequent procedures were performed using the DIG DNA Labeling & Detection Kit (Roche Diagnostics, Tokyo, Japan), according to the manufacturer's instructions. Signal intensity was densitometrically quantified as described previously.¹⁹

Western blot analysis

Western blot analyses were performed as described previously.¹³

Femtosecond laser-induced impulsive force loading assay

The methods for DRG neuron culture and its coculture with mast cells were as we described previously²¹ except that IC2 cells were used as mast cells. After 3 h, DRG neurite-IC2 cell cocultures were analysed by the femtosecond laser-induced impulsive force loading assay using our original device, as described previously.^{16, 17} Briefly, femtosecond laser pulses (570 nJ/pulse) were repeatedly focused in the culture medium through an objective lens (10×, numerical aperture = 0.12). The focal point of the first shot was positioned 100 μm from a targeted mast cell, the second shot then focused approximately 5 to 10 μm nearer the target (see Fig. 3a). The following shots were repeated in a similar manner until the mast cell detached. All the processes were video-recorded, and the distance between the last shot focal point and the target mast cell ($L_{\text{max to detach}}$) was measured. Approximately 100 mast cells were

selected randomly as targets, and were individually examined for their $L_{\max}$ to detach. In some experiments, IC2 cells were pre-cultured in the medium additionally containing various concentrations of either 9D2 or U04 for 2 h before the initiation of coculture. The cell suspension containing antibodies was transferred onto the DRG neurite network culture on the initiation of coculture.

Ca²⁺ indicator imaging assay

The Ca²⁺ indicator imaging assay was performed as described previously.^{15, 16}

Statistics analysis

Mathematical methods for the least-square regression analysis, Student's *t*-test and the χ^2 test were described previously.^{15, 16} A *P* value < 0.01 was considered to indicate significance.

Results

Increased expression of CADM1 in AD-like lesional mast cells

TNCB-sensitised BALB/c mice were repeatedly challenged with TNCB on the right ear auricle every 2 days for 3 weeks. After completion of the challenge, AD-like lesions had developed in the right auricle that was thickened due to fibrosis associated with marked infiltration of inflammatory cells (Figure S1; see Supporting Information). Alcian blue staining revealed that the number of mast cells in AD-like lesions ($321.2 \pm 31.2/\text{mm}^2$) was significantly larger than in the left ear auricles ($89.0 \pm 11.6/\text{mm}^2$, $P < 0.001$; Fig. 1), consistent with a previous report.¹⁰

We prepared fresh frozen sections from the right ear auricle to harvest skin mast cells using LCM and extracted RNA from these cells. For this we used toluidine blue, not alcian blue, to minimise RNA degradation during the staining procedure, because toluidine blue stained

mast cells more rapidly (~10 s). In toluidine blue-stained sections, skin mast cells were easily distinguishable from other cells by their metachromatic purple stain, and were selectively excised using LCM (Fig. 1a). Skin mast cells were also excised from frozen sections of the left ear as a non-AD-like lesional control. In addition, skin mast cells were excised from the ears removed 24 h after the first hapten challenge as an acute lesional (contact-hypersensitivity) control. Approximately 500 mast cells were harvested per right or left ear. Total RNA was extracted from the harvested cells, amounting to 10–20 ng per ear.

RNA extracted from LCM-harvested cells and BMMCs (3 ng each) was RT-PCR amplified using a primer set for either CADM1 or glyceraldehyde-3-phosphate dehydrogenase (G3PDH). Because the amount of PCR products was too small to be easily detected on agarose gels with ethidium bromide staining, the products were analysed by Southern blotting with gene-specific probes. Representative results from two mice are shown in Fig. 1b. We densitometrically quantified the Southern blotting results from all five mice and calculated the CADM1 expression levels relative to that of G3PDH. The mean CADM1 level in the AD-like lesional mast cells was three-fold higher than in the non-AD-like lesional and acute lesional mast cells, and as high as that in BMMCs (Fig. 1b).

Consistent results were obtained by immunohistochemistry. Paraffin-embedded sections of the right and left ear auricles were immunostained with an anti-CADM1 antibody, followed by alcian blue staining. In the left ear, alcian blue-stained mast cells were negative or faintly positive for CADM1, whereas in the right ear strong membranous staining for CADM1 was detected in the majority of AD-like lesional mast cells (Fig. 2).

CADM1 mediates DRG neurite–IC2 cell adhesion and communication

We previously reported that peripheral nerve fibres were CADM1-positive in various organs, including the lung and pancreas.^{22, 23} In AD-like lesional skin, CADM1-positive nerve fibres were occasionally observed in close proximity to CADM1-positive mast cells and invading into the thickened epidermis (Fig. 2c, d). Such findings were rarely detectable in non-lesional left ears. Because AD lesional skin is known to have an increased number of contacts between sensory nerve fibres and mast cells,^{4, 5} we investigated whether increased expression of CADM1 in AD-like lesional mast cells enhanced sensory nerve–mast cell interaction. Mouse DRG neurons were used as an example of sensory nerves. We used IC2 cells as mast cells lacking endogenous CADM1 and modified some to express CADM1 as abundantly as AD-like lesional mast cells by reference to the CADM1 level in BMMCs (IC2^{CADM1}) (Fig. 3b). We established a neurite network extending from the DRG neurons, and seeded either IC2 or IC2^{CADM1} cells onto the network. After 3 h of coculture, DRG neurite–IC2 cell adhesion was analysed by the femtosecond laser-induced impulsive force loading assay.^{16, 17} We repeatedly focused femtosecond laser pulses and measured the maximum focus–cell distance at which the target IC2 cell was detached ($L_{\text{max to detach}}$) (Fig. 3a). The median $L_{\text{max to detach}}$ of IC2^{CADM1} cells was significantly shorter than that of IC2 cells (Fig. 3c). When IC2^{CADM1} cells were preincubated with 9D2, a function-blocking antibody to CADM1, the $L_{\text{max to detach}}$ was increased in a 9D2-concentration-dependent manner (Fig. 3d). Such an effect was not detected on preincubation with U04, a control IgY.

After 12 h of the coculture, we loaded fluo-4 (ABD Bioquest), a fluorescent Ca^{2+} indicator, on all cultured cells, and then added scorpion venom (Sigma-Aldrich) to specifically stimulate DRG neurons. Immediately after addition of scorpion venom a sharp increase in $[\text{Ca}^{2+}]_i$ was detected in DRG neurites. Between 10 to 60 s following DRG activation, a substantial increase in $[\text{Ca}^{2+}]_i$ was detected in some but not all IC2 cells (Fig. 4). We

considered IC2 cells to be responding to DRG activation when $[Ca^{2+}]_i$ in IC2 cells increased more than 50% from the baseline. The percentage of IC2^{CADM1} cells responding (40.1%) was double that of IC2 cells (19.6%, $P < 0.001$) (Table 1). We next examined the response of IC2^{CADM1} cells to DRG activation in cocultures containing various concentrations of 9D2 or U04. The percentage of IC2^{CADM1} cells responding decreased in a 9D2-concentration-dependent manner, whereas it remained unchanged in the presence of U04 (Table 1). These results indicate that the amount of CADM1 on mast cells was a critical determinant of firm adhesion and efficient communication between DGR neurites and IC2 cells.

CADM1-expressing cells other than nerve and mast cells in AD-like lesions

When the AD-like lesions were immunostained with the CADM1 antibody, CADM1-specific cell-membranous signals were distributed not only in the dermis but also in the epidermis and hair follicles. A group of basal cells located in the elongated rete ridges strongly expressed CADM1, while some epidermal cells in the basal and prickle-cell layers expressed it at various levels (Figure S2). In the elongated hair follicles, a group of bulb cells and some bulge cells expressed CADM1 strongly and modestly, respectively (Figure S2). Some of these CADM1-positive epidermal and hair follicle cells appeared to be in contact with the CADM1-positive nerve fibres that ran along the dermis-epidermis interface and extended into the epidermis (depicted by large arrowheads in Figure S2). CADM1 immunohistochemistry of the non-AD-like lesional ears revealed that CADM1-positive staining was rarely detectable in epithelial cells (data not shown).

Discussion

In the present study, we found that CADM1 expression was elevated in AD-like lesional mast cells at both the mRNA and protein levels. Originally, we identified CADM1, formerly

named SgIGSF, as a mast-cell adhesion molecule whose expression was critically regulated by microphthalmia-transcription factor (MITF).¹³ Interestingly, MITF is necessary for mast cells to produce prostaglandin D₂ (PGD₂) through transactivation of the gene encoding a terminal synthase for PGD₂.²⁴ Additionally, Satoh *et al.* reported that PGD₂ plays an essential role in chronic allergic inflammation of the skin, including in AD.²⁵ These lines of evidence suggest that MITF function may be upregulated in AD lesional mast cells, leading to both enhanced PGD₂ production and increased CADM1 expression in mast cells. To test this hypothesis, we are currently investigating the molecular mechanisms of CADM1 upregulation in AD lesional mast cells.

Recent studies have demonstrated that sensory nerve–mast cell crosstalk plays a critical role in the pathophysiology of AD symptoms, especially pruritus, the hallmark of this disease.^{6,7} AD patients often scratch the lesional skin due to pruritus, and as a result, suffer from more severe pruritus and inflammation (“itch-scratch cycle”).⁷ In AD lesional skin, scratching appears to cause substance P release from sensory peptidergic neurons, subsequently evoking release of histamine and other mediators from mast cells via binding of substance P to neurokinin 1 receptors on mast cells.^{6,26} This mediator release, in turn, exacerbates itch by provoking afferent excitation of histamine-sensitive C-fibres and through augmenting neurogenic inflammation via the axon reflex.^{2,6,26} We showed here that increased expression of CADM1 in IC2 mast cells strengthened DRG neurite–IC2 mast cell adhesion and promoted DRG neurite-to-IC2 mast cell communication. Therefore, upregulation of CADM1 in lesional mast cells may be a key event in the establishment of the “itch-scratch cycle”. Thus, the present study identified CADM1 as a potential therapeutic target for blocking the “itch-scratch cycle”.

CADM1 upregulation also occurred in basal cells of elongated rete ridges as well as in bulge and bulb cells of elongated hair follicles. This finding was consistent with previous observations by Watt *et al.*, who reported that CADM1 is highly expressed in embryonic hair follicle placodes and actively proliferating adult follicle bulb cells.²⁷ Interestingly, we observed that many CADM1-positive nerve fibres ran along the dermis–epidermis interface and extended into the epidermis, with some in contact with CADM1-positive epidermal and hair follicle cells. Although we have at present not fully characterised these CADM1-positive skin cells, earlier anatomical studies demonstrated the presence of dense innervation around hair follicle cells and connections between sensory nerves and keratinocytes, melanocytes, Langerhans cells, or Merkel cells.²⁸⁻³² CADM1 upregulation in some or all of these skin cells may be another trigger of severe pruritus in AD through promoting functional interactions between nerves and these skin cells.

In addition to immunological aspects, psychological and neurological stresses contribute to a worsening condition, especially in adult patients with AD.^{1, 2} This fact indicates that both autonomic and sensory nerves are involved in AD pathogenesis. We have demonstrated that CADM1 promotes the sympathetic nerve–mast cell communication that is mediated by the action of neurite-derived substance P on neurokinin 1 receptors of mast cells.^{15, 33} In AD lesional skin, not only sensory nerve–mast cell communication, but also sympathetic nerve–mast cell communication, may be enhanced by a mechanism involving increased expression of CADM1 on mast cells. Further characterisation of CADM1 as a promoter of nerve–mast cell interaction in AD lesional skin will lead to a deeper understanding of the psychological aspects of AD pathogenesis and open a new avenue for better treatment of patients with AD.

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Figure legends

Fig. 1. Comparison of CADM1 mRNA levels in mast cells from AD-like and control ears. (a) In frozen sections of the ear of TNCB-induced AD and contact hypersensitivity model mice, skin mast cells were stained with toluidine blue (1), marked with a circular line (2), and cut out by LCM (3). (b) Total RNAs were extracted from LCM-harvested skin mast cells (non-AD-like lesional, AD-like lesional, and acute lesional) and BMMCs, and were RT-PCR-amplified, then analysed by Southern blotting with a specific probe for CADM1 or G3PDH. CADM1 band intensities were converted to densitometric values relative to G3PDH. The mean and SD were calculated from the data of 5 mice, and plotted in a bar graph with lines indicating SD. Expression levels of CADM1 mRNA are expressed as relative values normalized to 1 for the mean in non-lesional mast cells. *, $P < 0.01$ by *t*-test, when compared with the value of non-AD-like lesional mast cells.

Fig. 2. Immunohistochemistry of CADM1 in the ear of AD mouse model. Sections of the non-AD-like-lesional (a) and AD-like-lesional (b-d) skin were immunostained with the CADM1 antibody (brown), and then stained with alcian blue and nuclear fast red.

Arrowheads in (c) and (d) depict CADM1-positive nerve fibres. Original magnification, X 1000. Bar = 50 μ m.

Fig. 3. Femtosecond laser-induced impulsive force loading assay of DRG neurite-IC2 mast cell cocultures. (a) Schematic presentation of this assay. Femtosecond laser was repeatedly irradiated until a target IC2 cell detached from a neurite to measure $L_{\text{max to detach}}$. (b) Western blot analysis of CADM1 in IC2 cells and BMMCs. The blot was reprobed with anti- β -actin antibody to indicate the protein loading per lane. (c) Histograms of $L_{\text{max to detach}}$ of IC2 and IC2^{CADM1} cells in the coculture with DRG neurites. According to the least squares regression method, probability density distribution of $L_{\text{max to detach}}$ was approximated to one Gaussian curve (gray line). The median and SD of the Gaussian curve are shown above the histogram. (d) IC2 and IC2^{CADM1} cells were cocultured with DRG neurites in the absence (0) or presence (1, 3, or 10 μ g/ml) of 9D2 or U04, and histograms of $L_{\text{max to detach}}$ were made. Gaussian curves were approximated from the histograms, and the medians of the Gaussian curves were plotted in a bar graph with lines indicating SD. The leftmost two bars correspond to the histograms of (c). *, $P < 0.01$ by t -test, when compared with the value of IC2^{CADM1} cells in the absence of antibodies.

Fig. 4. Ca²⁺ indicator imaging assay of DRG neurite-IC2^{CADM1} cell cocultures. IC2^{CADM1} cells were cocultured with DRG neurites for 12 h and were then loaded with fluo-4. Cocultures were placed under observation through a confocal laser scanning microscope and were stimulated with scorpion venom (1 μ g/ml at $t = 0$). Before and after the addition of scorpion venom, fluo-4 fluorescence was detected every 3 seconds. Transmission and fluorescence images at the indicated time points are shown. The fluorescence intensity is displayed by the 256-color spectrum; red indicates a greater intensity than blue. The line graphs show the time traces of changes in fluo-4 fluorescence detected in the regions defined in the $t = 0$ panels;

two lines for DRG neurites (numbered N₁ and N₂) and four lines for IC-2^{CADM1} cells (numbered 1 to 4). Asterisks depict DRG neuron cell bodies.

Table 1. Response of IC2 and IC2^{CADM1} cells to scorpion venom-induced DRG activation in the coculture with DRG neurites.

Cell	Antibody (µg/ml) ^a		No. of cells		<i>P</i> value ^b
	9D2	U04	Responding	Not responding	
IC2	0	0	20 (19.6) ^c	82	< 0.001
IC2 ^{CADM1}	0	0	57 (40.1)	85	
	0.2	0	15 (27.8)	39	0.108
	2.0	0	13 (24.5)	40	0.0431
	10	0	9 (17.3)	43	0.0029
	0	10	22 (42.3)	30	0.785

^a IC2 or IC2^{CADM1} cells were cocultured with DRG neurites in the absence (0) or presence (0.2, 2.0, or 10 µg/ml) of 9D2 or U04. ^b By χ^2 test when compared with the value of IC-2^{CADM1} cells in the absence of 9D2 and U04. ^c Numbers in parentheses are the percentage of cells responding [= Responding/(Responding + Not responding) x 100 (%)].





