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Separation Force Measurements Reveal Different Types of Modulation of E-cadherin-based Adhesion by Nectin-1 and -3*

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Nectins are Ca^{2+}-independent cell adhesion molecules found at cadherin-based adherens junctions. We used a dual pipette assay that measures the forces required to separate cell doublets to determine how nectins affect the formation and strength of cell-cell adhesion. Less force was required to separate doublets of L cells expressing nectin-1 or nectin-3 than to separate doublets of E-cadherin-expressing cells. Heterodimers formed between cells expressing nectin-1 or nectin-3 adhered more strongly than homodimers. Nectin-3 that does not *trans*-interact with nectin-1 inhibited E-cadherin-mediated adhesion. However, the extracellular fragment of nectin-1 did not have an agonistic effect on E-cadherin-dependent cell adhesion when it *trans*-interacted with nectin-3, expressed at high levels in cells. In contrast, the extracellular fragment of nectin-3 had a significant agonistic effect on cadherin-based adhesion when it interacted with endogenous nectin-1, expressed at low levels in cells. Our results indicate that E-cadherin is the key molecule involved in cell adhesion and that the regulation of E-cadherin-based adhesion involving cellular nectin-1 *trans*-interacting with nectin-3 is qualitatively different from that involving cellular nectin-3 *trans*-interacting with nectin-1 and depends on the nectin levels expressed by cells.

Cadherin-based cell adhesion is a critical determinant of tissue architecture in developing and adult metazoan organisms (1–3). Cadherins (4–10) are single-pass, integral membrane proteins of 115–130 kDa. They belong to the family of Ca^{2+}-dependent cell adhesion molecules, which affect cell morphology, architecture, and function (3, 11–13). More than 80 cadherins have been identified to date, each of which is expressed in a wide variety of cells, including epithelial and non-epithelial cells (3, 13). Classical cadherins function as multiprotein complexes: the ectodomains mediate homophilic adhesive binding (2, 14), whereas the intracellular C-terminal domains associate with catenins at the site of adherens junctions (AJs),[1] forming cadherin-catenin complexes (15, 16). The β- and γ-catenins interact with the cytoplasmic tail of E-cadherin, forming a complex that is coupled to the actin cytoskeleton through α-catenin (also an actin-binding protein (8, 17)) via its interactions with a number of actin-binding proteins, such as α-actinin and vinculin, or by directly binding to actin itself (18–21). These complexes are the functional cell adhesive units (22). The interaction between cadherin and catenin also requires Ca^{2+} (22–26) and is regulated by GTPases (17, 27). The association of E-cadherin with the actin cytoskeleton through these peripheral membrane proteins strengthens the cell-cell adhesion activity of E-cadherin (2, 18).

Nectins belong to a new family of integral membrane proteins found in most AJs (28). Nectins share structural and sequence features and are characterized by three Ig-like domains in their extracellular portion. Nectins are widely expressed in tissues and cells (29–35), where they are located mainly at AJs (36). Cell surface nectins form homodimers involved in cell-cell adhesion by interacting in *trans* with other nectins (28). These calcium-independent *trans*-interactions can be homophilic or heterophilic (28, 36, 37). Isotypes of nectin-1, -2, and -3 are generated by alternative splicing and differ by their transmembrane regions and cytoplasmic tails (29–31, 33, 38). The α-isotype of nectin-1, -2, and -3 carries a C-terminal PDZ-binding domain that is able to interact with afadin/AF6, an F-actin-binding protein, to form the afadin/nectin complex (33, 36, 38–40). Nectin-1 is abundant in the brain, and nectin-2 and -3 are found in virtually all tissues, including the testis (41). The long form of afadin (l-afadin (28, 40)) contains an F-actin binding domain (28) and can interact with ZO-1, α-catenin, and ponsin (42) in the cytoplasm at the site of AJs. Nectins do not interact directly with cadherins, but these adhesion molecules can interact with each other via their cytoplasmic domain-associated proteins (43). Thus, afadin appears to mediate interactions between nectins and the cadherin-catenin system in AJs (36) and between nectins and junctional adhesion molecules in tight junctions (44). Recent studies have shown that the structural organization of cadherin-based AJs and tight junctions in polarized epithelia is disrupted in afadin −/− mice (45).

The precise mechanism by which the nectin-afadin complex affects cell adhesiveness is not known. Presumably, it exerts its effects via the cytoskeletal organization and polarization of epithelial cells. These results seem to suggest that cross-talk occurs between the nectin/afadin and cadherin/catenin systems to promote cell adhesion. To clarify this issue, we used a methodology that measures separation force to investigate the effect of nectins on adhesion strength in the presence and absence of E-cadherin. Two cells were put in contact by micromanipulation in suspension to form an adhesive doublet and then separated. The force required to separate the two cells was meas-

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[1] The abbreviations used are: AJ, adherens junction; N1L, nectin-1; N3L, nectin-3; EL cells, E-cadherin-expressing cells; nN, nanonewtons.

ured on several cells containing various amounts of nectins and/or E-cadherin. We found that the force necessary to separate doublets of L cells expressing nectin-1 (N1L) or nectin-3 (N3L) was lower than that required to separate E-cadherin-expressing (EL) doublets. When cells expressed only E-cadherin or both E-cadherin and nectin-1, the forces required for separation were similar, suggesting that, in this dual pipette assay, the presence of nectin-1 does not modify or improve the "adhesiveness" of cells. In contrast, we observed that nectin-3 inhibited E-cadherin-adhesion activity when it was not *trans*-interacting with nectin-1. Our results indicate different modulation of E-cadherin-based adhesion by nectin-1 and nectin-3.

EXPERIMENTAL PROCEDURES

Antibodies and Reagents—DECMA-1 (rat anti-E-cadherin) antibody and phalloidin-fluorescein isothiocyanate were purchased from Sigma. Rabbit anti-human nectin-1α and rabbit anti-mouse nectin-3α polyclonal antibodies were produced as described previously (36, 38). Secondary antibodies coupled to Texas red or fluorescein were purchased from Jackson ImmunoResearch Laboratories. The extracellular fragments of nectin-1 and nectin-3 fused to the Fc portion of human IgG (Nef-1 and Nef-3, respectively) were produced as described previously (46).

Cell Lines and Reagents—N1L, N1ΔCL, and N3L clones are mouse L fibroblasts stably transfected clones expressing nectin-1, cytoplasmic deleted nectin-1ΔC (carrying the first 514aa), or nectin-3, respectively. They were described previously in Refs. 36 and 38. L fibroblasts stably transfected to express E-cadherin (EL cells) were produced as described previously (47). EL cells expressing nectin-1 and FLAG-nectin-1 (clones N1EL#4 and N1EL#8, respectively) were produced as described previously (44). N3EL (#2) cells expressing E-cadherin and FLAG-nectin-3 were produced as described in a previous study (48).

Tissue Culture and Cell Dissociation—Cells were cultured at 37 °C in a humidified incubator in a 5% CO_2-95% air atmosphere. EL cells were maintained in culture medium containing Dulbecco's modified Eagle's medium (Invitrogen) supplemented with 10% fetal bovine serum (Invitrogen), penicillin (100 IU/ml), streptomycin (100 μg/ml), and 2 mM L-glutamine (Sigma). N1L and N1ΔCL cells were grown in culture medium supplemented with 500 μg/ml G418. N1EL#8 cells were grown in culture medium supplemented with 500 μg/ml hygromycin (Clontech). N3L, N3EL#2, and N1EL#4 cells were grown in culture medium supplemented with 5 μg/ml puromycin. Confluent cultures were routinely treated with 0.05% trypsin and 0.02% EDTA (trypsin-EDTA treatment, Invitrogen) at 37 °C for 5 min and seeded at a sufficiently high density to yield a nearly confluent culture 18–24 h later.

To ensure that E-cadherin and nectins were not lost from their surfaces, completely dispersed cell suspensions were obtained by treating monolayers with 0.01% trypsin XI (Sigma) in HMF (magnesium-free HEPES-based buffer containing 10 mM calcium, pH 7.4 (49)). After centrifugation, the cell suspension was gently suspended in HCMF (calcium- and magnesium-free, HEPES-based) to yield isolated cells. The cells were then centrifuged and resuspended in working medium (CO_2-independent medium (Invitrogen) supplemented with 1% fetal bovine serum) and used immediately in the dual pipette assay. Alternatively, N1EL cell monolayers were treated with the trypsin-EDTA solution to yield suspended cells lacking cadherins at their surface but still retaining nectins (36).

Microscopy—The expression of E-cadherin and nectins was monitored by immunofluorescence microscopy in cells cultured on glass coverslips as described previously (50). Preparations were observed by epifluorescence microscopy, using a Leica DMRBE microscope equipped with a cooled charge-coupled device camera (Hamamatsu C5985). Data were acquired using a Power Macintosh workstation and IP-Lab software. The E-cadherin and nectins expressed at the surface of living cells were analyzed by flow cytometry as described before (51). Cell suspensions were prepared in a way to preserve E-cadherin at their surface and incubated in cold Dulbecco's modified Eagle's medium with a specific antibody directed against the extracellular domain of E-cadherin or against the cytoplasmic domain of nectins.

Quantification of E-cadherin and Nectin Levels in Cells—Cell monolayers were extracted and subjected to Western blot analysis as described previously (50) with antibodies directed against E-cadherin, nectin-1, and nectin-3. Bound antibodies were detected using the ECL system (Amersham Biosciences). Signals were quantified using National Institutes of Health Image software. Cellular E-cadherin and nectins were quantified by using standard curves built using a linear range of concentrations of the recombinant MBP-Nectin-1, MBP-Nectin-3, and glutathione *S*-transferase-E-cadherin (cytoplasmic domain) proteins as described previously (52).

Micropipette Preparation—Micropipettes were made from borosilicate glass capillary GC100–15 tubing (1.0-mm outside diameter × 0.58-mm wall thickness × 7-cm length, Harvard apparatus Ltd., Kent, UK) using a pipette puller (Sutter instruments, model P-2000) and a homemade Microforge, such that their inner diameter was 5.0–6.5 μm. Pipettes were coated with bovine serum albumin. Prior to experiments, the pipettes were filled with isotonic sucrose solution (300 mosmol).

Measurement of Separation Forces—Separation force measurements were done as described previously (53). The experimental technique is based on a modification of a dual-micropipette assay (54), which permits manipulation of individual cells to form controlled contacts. Briefly, the experiments were performed on the stage of a Leica inverted epifluorescence microscope positioned on an anti-vibration platform and equipped with a cooled charge-coupled device Hamamatsu C5985 or Nikon Coolpix 5000 camera, a digitally controlled heating stage and 10× and 63× objectives. The incubation chamber consisted of the bottom half of a 90-mm Petri dish previously coated with bovine serum albumin for 12 h. All experiments were performed at 37 °C. An inverted bottom of a second Petri dish of the same size was used to cover the stage and to keep humidity and temperature constant. Two micropipettes were positioned in a dual entry chamber mounted on the microscope stage. Each was held in its own micromanipulator and connected to a combined hydraulic/pneumatic system, making it possible to control the aspiration applied to the cells. Two cells (expressing either the same or different adhesive proteins) were gently aspirated, one on the tip of each pipette (to form homodimers or heterodimers (Fig. 1*A*)), and brought into contact through the use of the micromanipulators (Fig. 1*B*). For experiments performed on the validation of the initial contact, we measured the force require to separate doublets (see below) immediately after putting the cells in contact. Alternatively, cells were allowed to remain in contact for a designated period of time. To separate cells in contact, the aspiration in the right pipette was maintained at a level sufficiently high to hold one of the two cells in contact tightly. The aspiration in the left pipette was increased in steps, the magnitudes of which were measured with a pressure sensor (Validyne: model DP103-38, 0–50,000 Pa). After each step, the pipettes were moved apart in an effort to detach the adherent cells from one another (Fig. 1*C*). A doublet pulled intact from the left pipette was moved back to the orifice of the pipette, and the cycle was repeated until the level of aspiration reached in the left pipette was sufficient to pull one cell apart from the other (Fig. 1*C*). The aspiration was monitored continuously during the separation process, and the values recorded for each of the last two cycles in the series (P_{n-1} and P_n) were used to calculate the separation force (F) for each doublet using the equation: $F = \pi(d/2)^2 (P_{n-1} + P_n)/2$, where d is the internal diameter of left pipette. Results from 30–50 measurements were used to obtain the mean force of separation for a specific contact time in each experiment. The separation force is referred to as F.

RESULTS

Determination of E-cadherin and Nectin Levels in Stably Transfected L Cells—The mouse L fibroblasts stably transfected to express E-cadherin, nectins, or both were analyzed by immunofluorescent staining and Western blotting. Cadherins and nectins were found at the surface and recruited to cell boundaries (Fig. 2). When both molecules were expressed in the same cells, they colocalized at the sites of cell-cell contact, as shown in the merged images. E-cadherin and nectin levels were measured in the various clones (Table I). EL, N1EL#4, N1EL#8, and N3EL#2 expressed a similar amount of E-cadherin (around 1×10^6 molecules/cell), whereas L, N1L, N1L#3, N1L#5, N1L#6, N1ΔCL, and N3L did not express a detectable amount of this molecule. All these clones contained nectins at various levels of expression.

Nectins Generate Weaker Adhesion Than E-cadherin—We used an approach that gives an estimate of the strength of adhesion between cells mediated by nectins and E-cadherin. Cell adhesion was initiated by using micropipettes, and we then measured the forces required to separate them. We have previously observed that a strong force is required to separate sarcoma S180 cells expressing E-cadherin after 4 min of contact. The amount of force required increases with the time of

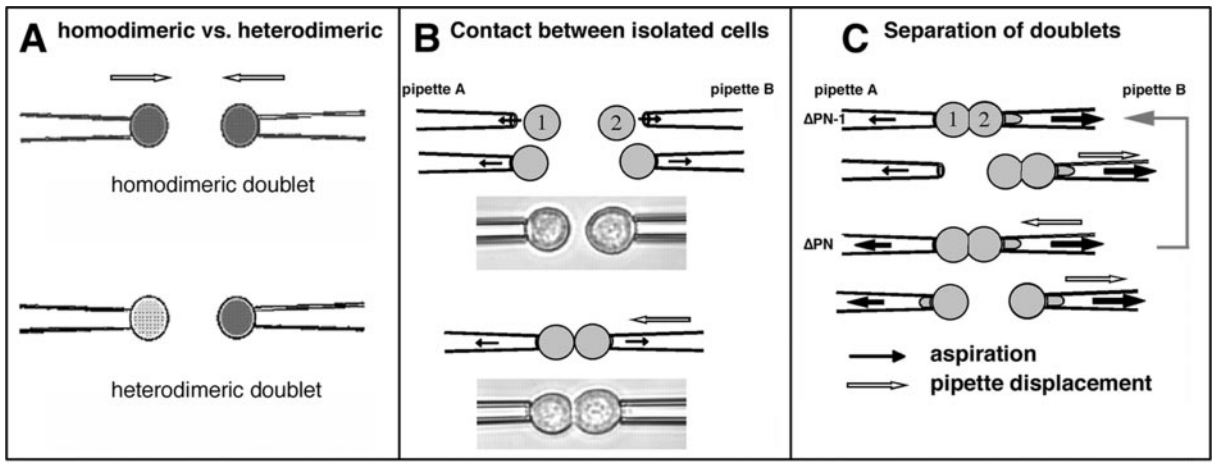

FIG. 1. **Micromanipulation of isolated cells to form homodimeric or heterodimeric doublets and schematic representation of the procedure used to form and to separate adhering cells.** *A*, we brought two L cells expressing either E-cadherin, nectin, or both molecules into contact, by micromanipulation. Homodimeric doublets were formed from cells expressing the same adhesive proteins: for example, nectin-1 (N1L), nectin-1ΔC (N1ΔCL), nectin-3 (N3L), E-cadherin (EL), nectin-1-E-cadherin (N1EL), and nectin-3-E-cadherin (N3EL). Heterodimeric doublets were formed from L cells expressing different adhesive proteins: nectin-1 and nectin-3 (N1L-N3L), and nectin-1-E-cadherin and nectin-3-E-cadherin (N1EL-N3EL). *B*, the two cells were held under weak aspiration by micropipettes and placed in contact for different periods of time (between 0.5 and 60 min). *C*, cell #2 was held by micropipette B under strong aspiration. The aspiration applied to cell #*1* was increased, and the micropipette displaced, step by step, until the adherent cells separated. *Black arrows* represent the aspiration applied to cells; the *size of arrows* represents the magnitude of the aspiration force during the separation procedure. *White arrows* represent the displacement of the pipette during the experiment.

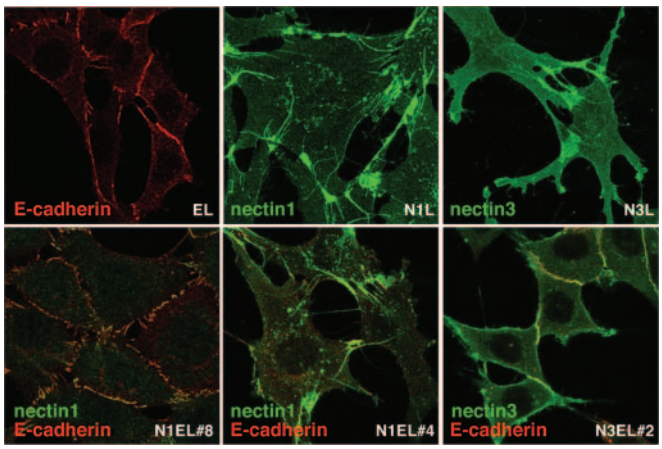

FIG. 2. **Location of E-cadherin and nectins in cells.** Mouse L fibroblasts stably transfected to express E-cadherin, nectins, or both were processed for immunofluorescent staining with specific antibodies. *Top*, *left* to *right*: EL, N1L, and N3L cells express full-length E-cadherin, nectin-1α, and nectin-3α, respectively. E-cadherin and nectins were found at the surface and at cell boundaries. *Bottom*, *left* to *right*: N1EL#8, N1EL#4, and N3EL#2 cells express E-cadherin together with FLAG-nectin-1α, full-length nectin-1α, and FLAG-nectin-3α, respectively. E-cadherin and nectins colocalized at the site of cell-cell contact as shown in the *merged images*.

TABLE I
Levels of nectins and E-cadherin in cells

Cell lines	Nectin	E-cadherin
	molecules/cell	
L	1×10^4	nd[a]
EL	1×10^4	1×10^6
N1L	5.7×10^6	nd
N1L#3	1.4×10^6	nd
N1L#5	0.3×10^6	nd
N1L#6	1×10^6	nd
N1ΔCL	3.5×10^6	nd
N3L	6.1×10^6	nd
N1EL#8	0.9×10^6	1×10^6
N1EL#4	5.1×10^6	1.1×10^6
N3EL#2	7.9×10^6	0.9×10^6

[a] nd, not detected.

contact and the amount of cadherin expressed at the surface (55).

In this study, we compared the mean separation force obtained after 4 min of contact between homodimeric EL, N1L#6, N1L, N1ΔCL, and N3L doublets and heterodimeric N1L-N3L doublets (Fig. 3). Although EL and N1L#6 cells expressed similar amount of adhesion molecules at their surface (1×10^6 molecules of E-cadherin or nectin-1, respectively, per cell), the forces measured to separate EL doublets (29.3 nN) were higher than those measured for N1L doublets (1.5 nN). In addition, when a large amount of nectin-1 or nectin-3 are expressed at the cell surface (around 6×10^6 molecules/cell), the forces required to separate N1L, N3L, and N1L-N3L doublets (2.2, 1.3, and 7.7 nN, respectively) were still weaker than those required to separate EL doublets. This indicates that nectins generate weaker interactions than E-cadherin. We observed that greater force was required to separate N1L-N3L (7.7 nN) heterodimers than to separate the N1L or N3L homodimers (2.2 and 1.3 nN, respectively). When cells expressed 3.5×10^6 molecules of nectin-1 lacking its cytoplasmic domain (N1ΔCL cells), the separation force was 1 nN, which is statistically weaker than that measured for N1L (2.2 nN) and N1L#6 (1.56 nN) doublets. This suggests that the cytoplasmic domain of nectin may be involved in the control of nectin-based adhesion strength.

We then compared separation force measured for EL and N1L doublets after 4 and 60 min of contact (Fig. 4). The cadherin-mediated adhesion in EL cells was time-dependent (29 nN after 4 min and 59 nN after 60 min of contact; Fig. 4). In contrast, no statistically significant difference was observed for separation force measured for N1L doublets after 4 and 60 min of contact (2.2 and 3.7 nN, respectively).

It has been previously shown that the expression levels of adhesion molecules at the cell surface modulate separation force (55). We measured separation force for parental L cells and for various nectin-1-expressing clones (N1L, N1L#5, N1L#6, and N1L#3; Fig. 5*A*). L doublets expressing very low levels of nectin-1 did not display measurable separation force, whereas N1L#5, N1L#6, N1L#3, and N1L doublets displayed significant separation force of 1.16, 1.54, 1.65, and 2.24 nN, respectively (Fig. 5*B*). These results indicate that the separation force measured varied with expression levels of nectin-1 at the cell surface. However, we observed that 1) no detectable

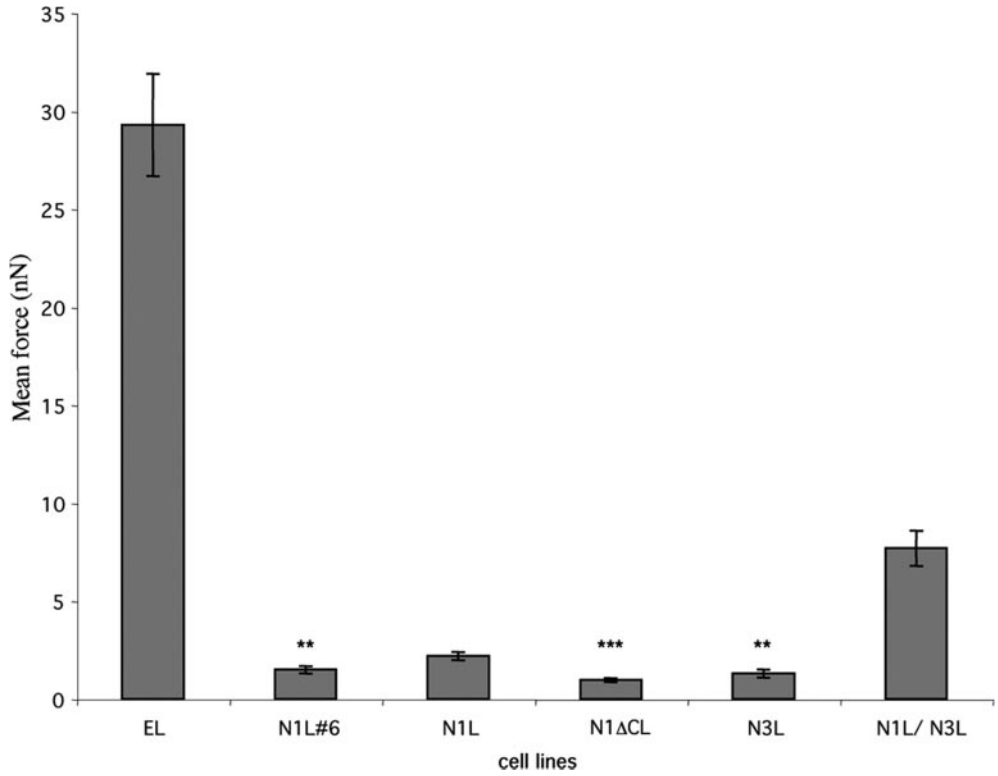

FIG. 3. **Measurement of separation force after 4 min of contact.** The force required to separate homodimeric N1L, N1L#6, and N3L doublets, and heterodimeric N1L-N3L doublets was weaker than that required to separate homodimeric EL doublets. The N1ΔCL doublets (expressing nectin-1 lacking the cytoplasmic domain) exhibited a slightly weaker separation force than N1L doublets. The *asterisks* indicate significant differences relative to N1L cells; **, $p \leq 0.001$; ***, $p \leq 0.0001$ (Student's t test).

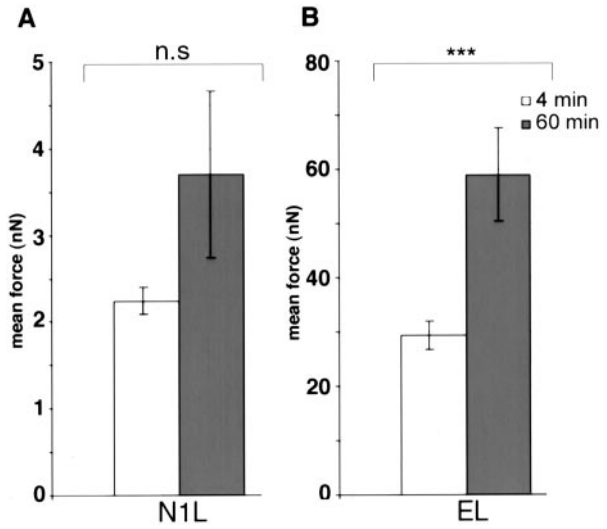

FIG. 4. **Effect of time of contact on separation force.** The separation force measured for N1L doublets (*A*) and EL doublets (*B*) increased with time of contact. However, the rate at which the separation force increased was slower for N1L doublets than for EL doublets. The *asterisks* indicate significant difference with $p \leq 0.0001$ (Student's t test). *ns*, not statistically different.

force can be measured for low levels of nectins (10,000 nectin-1 molecules) and 2) there was no statistically significant difference between force measured for N1L#5, -#6, and -#3 expressing from 0.32 to 1.4 millions of nectin-1. This indicates that nectins generate low adhesion strength, and the measured values for these clones were close to the detection limit of the system enabling us to determine a detailed dose response curve of separation force as a function of nectin levels.

Distinct Effects of trans-Interacting and Non-trans-interacting Nectins on E-cadherin-based Adhesion—EL, N1EL#8, and N1EL#4 cells expressed a similar level of E-cadherin at their surface (10^6 molecules/cell), whereas they exhibited increased levels of nectins (10^4, 0.9×10^6, and 5.1×10^6 molecules/cell of nectin^{-1}, respectively). However, the three types of doublets displayed a similar separation force after 4 min of contact. In contrast, N3EL#2 doublets (with 10^6 E-cadherin molecules and 7.9×10^6 nectin-3 molecules/cell) exhibited a significantly lower separation force (7.2 nN) compared with EL and N1EL doublets (Fig. 6*A*). This suggests that high levels of nectins, non-*trans*-interacting nectins (nectins that do not interact with other nectins), and the type of nectins affected adhesion strength.

The separation force measured for heterodimers formed between N1EL#4 or N1EL#8 cells and N3EL#2 cells were significantly higher than those measured for N3EL#2 homodimers (Fig. 6*A*). This indicates that nectin-3 inhibited the E-cadherin-based adhesion when it was unable to interact with nectin-1. Furthermore, the heterodimer formed between N1EL#4 cells and N3EL#2 cells (expressing 5.1×10^6 molecules/cell nectin-1 and 7.9×10^6 molecules/cell nectin-3, respectively) displayed a separation force of 29.7 nN, which is significantly higher than that formed by N1EL#8 and N3EL#2 (23.2 nN; expressing 1×10^6 of nectin-1 and 7.9×10^6 of nectin-3, respectively; Fig. 6*A*). This suggests that the ratio between *trans*-interacting and non-*trans*-interacting nectins modulates the E-cadherin-based adhesion strength.

Effects of Nectins on the Initiation of E-cadherin-based Adhesion—trans-Interaction of nectins has been proposed to recruit E-cadherin and to induce the formation of adherens junctions (28). To test this hypothesis, another set of separation force measurements was performed 1) at two early steps of cell adhesion, the validation of the initial cell contact and after 30 s of contact, and 2) at intermediary times of 2 and 4 min of cell contact. At initial contact, EL, N1EL, and N3EL homodimers and N3EL-N1EL heterodimers displayed forces of 16.4, 11.7,

A

	separation force at 4 min (nN)	Nectin-1 molecules
L	0	10 000
N1L#5	1.16	320000
N1L# 6	1.54	1000000
N1L#3	1.65	1400000
NIL	2.24	5700000

B

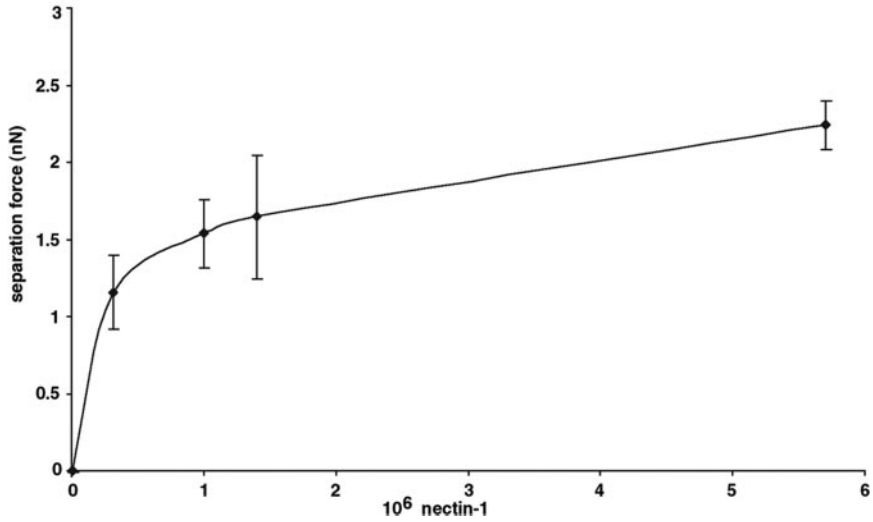

FIG. 5. **Effect of nectin levels on separation force.** *A*, separation force was measured after 4 min of contact for L, N1L#3, #5, #6, and N1L doublets expressing increased levels of nectins. *B*, the separation force measured for the nectin-1-expressing doublets increased with the nectin expression level.

6.5, and 10.4 nN, respectively, reflecting an inhibitory effect of nectin-1 and nectin-3 on the initiation of E-cadherin-based adhesion (Fig. 6*B*).

Homodimeric EL, N1EL, and heterodimeric N3EL-N1EL doublets displayed a 17%, 44, and 81% increase in the separation force measured after 30 s of contact (19.2, 16.9, and 18.9 nN, respectively) compared with that measured at the initial contact. In contrast, homodimeric N3EL doublets exhibited an unchanged separation force of 6.2 nN at 30 s (Fig. 6*B*). This observation suggests that the presence of nectin-1 or the trans-interacting nectin-3 with nectin-1 promotes higher velocity in the developing E-cadherin adhesion compared with non-*trans*-interacting nectins.

From 30 s to 2 min of contact, the force measured for EL, N1EL, and N3EL doublets increased of 31%, 19, and 82% (24.4, 20.1, and 11.6 nN, respectively), whereas the heterodimeric N3EL-N1EL doublet value remained unchanged with 18.2 nN measured (Fig. 6*B*). From 2 to 4 min of contact, the force measured for EL and N1EL continued to slightly increase with time; a 8 and 30% increment was obtained (26.4 and 26.1 nN, respectively, measured at 4 min). In contrast, the values obtained for N3EL doublets and heterodimeric N3EL-N1EL doublets remained unchanged (Fig. 6*B*).

Taken together, the results indicate that the presence of a large amount of nectins in N1EL cells did not promote higher initial adhesion strength compared with EL doublets. However, the rate at which the separation force increased between the initial contact and 4 min was significantly higher in cells expressing both nectin and E-cadherin than in EL doublets.

To analyze further the effect of non-*trans*-interacting nectins and the effect of homo *versus* hetero-*trans*-interacting nectins on the E-cadherin-based adhesion, we measured the separation force of EL and N3EL doublets in the presence of soluble fragments of nectin-3 (Nef-3) and nectin-1 (Nef-1), respectively. When Nef-3 (4 μM) was added to EL cells (expressing 10^4 endogenous nectin-1 molecules/cell), the separation force was 51 nN compared with 29 nN in control conditions (Fig. 7*A*). In contrast, Nef-1 slightly reduced the separation force of N3EL cells (Fig. 7*B*).

DISCUSSION

It has been reported, that in cultured cadherin-deficient L cells, the expression of nectin-1 is able to promote cell-cell adhesion in a gyratory aggregation assay (36). However, the cell-cell adhesion activity of nectin-1 was estimated to be weaker than that of E-cadherin: N1L aggregates were easily dispersed by pipetting, and phase contrast microscopy showed that the cell boundaries between N1L cells were not compacted compared with those between EL cells (46). Our results confirm that cell adhesion mediated by nectins is weaker than that mediated by E-cadherin. For similar or higher levels of expression, nectin-1 and nectin-3 produce 10- to 20-fold weaker adhesion than E-cadherin in EL doublets. Our dual micropipette assay made it possible to quantify adhesion in heterodimeric doublets (formed between two cells expressing different type of adhesion molecules). N3L-N1L heterodimeric doublets displayed stronger adhesion strength than N1L-N1L or N3L-N3L homodimeric doublets. Our results are consistent with earlier studies, showing that smaller aggregates are obtained when the cell aggregation assay is performed with cells expressing nectin-1, -2, or -3 alone than when it is performed with a mixture of cells expressing nectin-1 and -3 (38, 46). Indeed, the first Ig-loop of nectin-3-Fc interacts with nectin-1 with a high affinity (K_D 4 nM (56)). Finally, the *trans*-interaction of nectin-1 with nectin-3 is the nectin combination that induces filopodia and lamellipodia most strongly (57).

We found that the force required to separate doublets of cells expressing the full-length nectin-1 (N1L and N1L#3, -#5, and -#6) was higher than that required to separate doublets of cells expressing nectin-1 with a deletion of the cytoplasmic domain. However, it was more difficult to obtain reliable and reproducible measurements for N1ΔCL doublets than for nectin-1 ex-

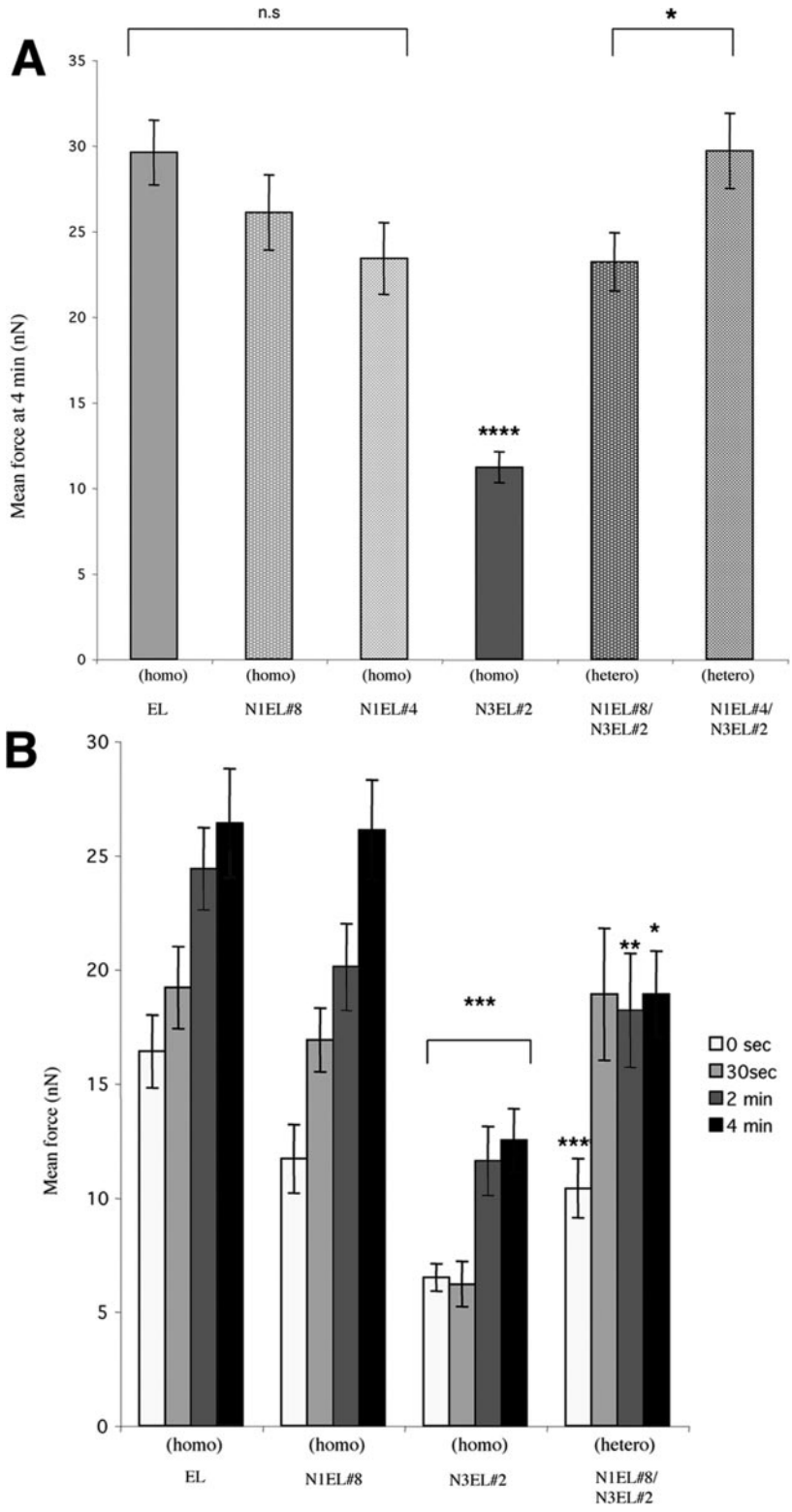

FIG. 6. **Separation forces for homo- and heterodimeric doublets.** *A*, after 4 min of cell contact, the separation force was lower for homodimeric N3EL#2 doublets than for homodimeric EL and N1EL (*#8* and *#4*) doublets. In contrast, the separation force of heterodimeric doublets composed of N1EL#4 cells and N3EL#2 cells was similar to the separation force of EL and N1EL#8 homodimeric doublets. The *asterisks* indicate significant differences of N1EL#4/N3EL#2 doublets relative to N1EL#8/N3EL#2 (* $p \leq 0.01$), and N3EL#2 doublets relative to EL and N1EL doublets (****, $p \leq 0.00001$; Student's *t* test). *ns*, not statistically different. *B*, effect of contact time on separation force for homodimeric and heterodimeric doublets. The separation force was measured just after initiation of the contact (*white bars*), after 30 s (*light gray bars*), 2 min (*dark gray bars*), and 4 min (*black bars*) of contact. The *asterisks* indicate significant differences of N3EL values relative to the other clones at the four time points, and of N1EL#8/N3EL#2 relative to EL doublets at the corresponding time points and for N1EL#8/N3EL#2 relative to N1EL#8 at 4 min (*, **, and *** for $p \leq$ 0.01, 0.001, and 0.0001, respectively; Student's *t* test).

pressing doublets after 4 min of contact; the measured values were close to the detection limit of the system for N1ΔCL doublets. The cytoplasmic domain of nectin-1 is known to interact with the actin cytoskeleton via afadin and catenins. We have previously observed that drugs that interfere with actin polymerization strongly decrease (from 40 to 10 nN) the separation force for E-cadherin-expressing S180 sarcoma cell doublets (55). With these drugs, the adhesion developed between cells occurs mostly via the interaction between the extracellular domains of cadherins at the intercellular contact with no connection to the actin cytoskeleton. When cells expressed nectins, the separation force was considerably weaker than for EL cells. Furthermore, deletion of the cytoplasmic domain of nectin-1 did not dramatically reduce the force needed to separate adherent N1L cells, suggesting that adhesion strength is mostly dependent on the *trans*-interaction between the extracellular domains of nectins and does not involved the interaction of nectins with cytoplasmic connectors in agreement with previous studies (58). These results also suggest that nectins are not as strongly connected to the cytoskeleton as is E-

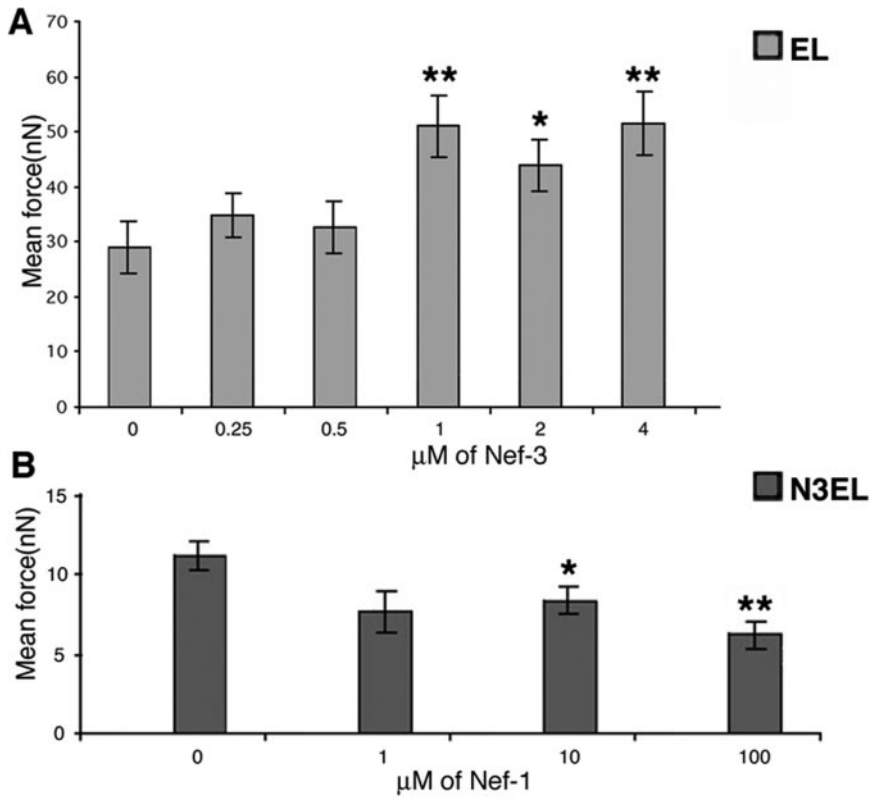

FIG. 7. **Agonistic and antagonistic effect of soluble extracellular fragment of nectin-1 and -3 in the E-cadherin-based adhesion.** *A*, after 4 min of contact, EL cells were separated in the presence of various concentrations of the soluble extracellular fragment of nectin-3 fused to the Fc portion of the human IgG (Nef-3). *B*, after 4 min of contact, N3EL#2 cells were separated in presence of the extracellular fragment of nectin-1 fused to the Fc portion of the human IgG (Nef-1). *Asterisks* indicate differences with respect to the control; *, $p < 0.01$ and **, $p \leq 0.001$.

cadherin. Furthermore, the fact that the separation force was less affected by contact time in N1L cells suggests that actin cytoskeleton reorganization is not required for the development of nectin-based adhesion.

In our work, N1EL#4 and N3EL#2 cells, which express a large amount of nectin-1 and nectin-3, respectively, adhered less efficiently than N1EL#8, which expresses less nectin-1 at 4 min. This was particularly obvious for N3EL cells. Several explanations could account for this effect. First, it is possible that the huge amount of nectin in cells, about 5- to 10-fold higher than that of E-cadherin, exerts a steric inhibitory effect by diluting the E-cadherin molecules at the cell-cell contact zone. Second, nectin, especially nectin-3, that does not immediately *trans*-interact with nectin-1 could inhibit the E-cadherin-mediated adhesion of N3EL cells. Interestingly, when we reduced the amount of non *trans*-interacting nectin-3 (*i.e.* when N3EL cells were put in contact with N1EL#4 cell, which express more nectin-1), the adhesion strength increased significantly (compare the separation force for N1EL#4-N3EL and N1EL#8-N3EL heterodimers). These observations strongly suggest that non-*trans*-interacting nectin-3 with nectin-1 inhibits E-cadherin function.

We found that adherent doublets expressing either cadherins or cadherins and nectin-1 displayed similar separation force. Our results also suggest that E-cadherin generates adhesion strength, whereas nectins may speed up the development of nascent cell-cell adhesion as revealed by fact that the separation force increased more quickly between the initial contact and 30 s to 4 min in homodimers and heterodimers of nectin-expressing EL doublets than in EL doublets.

It has recently been found that non-*trans*-interacting nectin-1 inhibits the E-cadherin-induced activation of Rac as well as formation of adherens junctions and that this inhibitory effect is suppressed by the *trans*-interaction of nectin-1 with Nef-3 (48). Based on these observations, we would expect that when cells expressing both cadherin and nectin-1 are incubated with Nef-3 they display a higher separation force compared with in control conditions. Consistent with this hypothesis, we observed that Nef-3 had an agonistic effect on the E-cadherin-based adhesion strength in EL cells. These results indicate that, when cells express low amounts of nectin-1 (EL cells; 10^4 molecules nectin per cell), their *trans*-activation with Nef-3 has an agonistic effect on the E-cadherin-based adhesion strength. This agonistic effect of Nef-3 is consistent with previous observations showing that Nef-3-coated microbeads promoted the recruitment of the nectin-1-afadin complex and the transport of the E-cadherin-catenin complex to bead-cell contact sites in N1EL cells (46). Moreover the *trans*-interaction of Nef-3 with nectin-1 activates Cdc42 and Rac, which might in turn increase E-cadherin-based adhesion strength at the sites of cell-cell contact (57, 59).

The addition of Nef-1 in N3EL doublets decreased separation force, whereas the force measured for heterodimeric N1EL-N3EL doublets was higher than that measured for N3EL doublets. Therefore, the inhibitory effect of non-*trans*-interacting nectin-3 with nectin-1 on E-cadherin-based adhesion may only be reverted when the molecules are anchored to the membrane via nectin-1 from the apposed cells but not via a soluble Nef-1, whereas the agonistic effect of *trans*-interacting nectin-1 does not require the anchoring of molecules to the membrane. Some previous results have suggested a qualitative difference between nectin-1 and nectin-3 functions. Kawakatsu *et al.* (57) showed that filopodia and lamelipodia were induced when N1L and N3L cells were placed on coverslips coated with Nef-3 and Nef-1, respectively. However, the shape of N1L cells on Nef-3 appeared slightly different from that of N3L cells on Nef-1.[2] On the basis of our results, we propose that intracellular signal transduction involving cellular nectin-1 *trans*-interacting with Nef-3 differs from that involving cellular nectin-3 *trans*-interacting with Nef-1 and exerts specific effects on the E-cadherin-based adhesion.

[2] T. Kawakatsu and Y. Takai, unpublished data.

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