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Involvement of up-regulated Necl-5/Tage4/PVR/CD155 in the loss of contact inhibition in transformed NIH3T3 cells

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Abstract

Normal cells show contact inhibition of cell movement and proliferation, but this is lost following transformation. We found that Necl-5, originally identified as a poliovirus receptor and up-regulated in many cancer cells, enhances growth factor-induced cell movement and proliferation. We showed that when cells contact other cells, Necl-5 interacts in *trans* with nectin-3 and is removed by endocytosis from the cell surface, resulting in a reduction of cell movement and proliferation. We show here that up-regulation of the gene encoding Necl-5 by the oncogene V12-Ki-Ras causes enhanced cell movement and proliferation. Upon cell–cell contact, *de novo* synthesis of Necl-5 exceeds the rate of Necl-5 endocytosis, eventually resulting in a net increase in the amount of Necl-5 at the cell surface. In addition, expression of the gene encoding nectin-3 is markedly reduced in transformed cells. Thus, up-regulation of Necl-5 following transformation contributes to the loss of contact inhibition in transformed cells.
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Keywords: Necl-5; Tage4; Poliovirus receptor; CD155; Nectin; Cell movement; Cell proliferation; Contact inhibition; Ras; Transformation

Cells move and proliferate until they come into contact with other cells. Upon cell–cell contact, cell–cell adhesion occurs, and movement and proliferation stop. This phenomenon is known as contact inhibition of cell movement and proliferation [1,2], and has been shown to be implicated in a variety of critical events such as organogenesis and tissue repair [3,4]. When cells are transformed, they loose contact inhibition, causing abnormal cell proliferation, invasion, and metastasis [5,6]. However, the principal mechanisms of these physiological and pathological phenomena are unknown.

We found that an Ig-like molecule Necl-5/Tage4/poliovirus receptor (PVR)/CD155 co-localizes with integrin αVβ3 at the leading edges of moving cells and enhances growth factor-induced cell movement and proliferation [7,8]. Human PVR/CD155 was originally identified as a poliovirus receptor [9,10], whereas rodent Tage4 was originally identified as the product of a gene overexpressed in rodent colon carcinoma [11]. PVR/CD155 is also overexpressed in many human cancer cells [12,13]. This molecule, with four nomenclatures, is re-named nectin-like molecule-5, Necl-5 [14]. Necl-5 does not show homophilic cell–cell adhesion activity, but it interacts in *trans* with nectin-3, a member of the Ig-like nectin family that is a Ca^{2+}-independent cell–cell adhesion molecule and cooperatively forms adherens junctions with cadherin [14,15]. When cells contact other cells, Necl-5 is removed from the cell surface by clathrin-dependent endocytosis, due to its *trans*-interaction with nectin-3 [16]; however, when cells do not contact other cells, the gene encoding Necl-5 is up-regulated by

Abbreviations: Necl, nectin-like molecule; Nef, the fusion protein containing the extracellular region of nectin fused to the Fc portion of human IgG; pAb, polyclonal antibody; siRNA, small interfering RNA; PVR, poliovirus receptor; V12-Ki-Ras-NIH3T3 cells, NIH3T3 cells transformed by an oncogenic Ki-Ras.

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growth factor-induced signaling [17]. Up-regulation of Necl-5 enhances growth factor-induced cell movement and proliferation, whereas down-regulation of Necl-5 reduces these processes [16]. We have proposed that Necl-5 has a role, at least in part, in the contact inhibition of cell movement and proliferation [16].

We recently found that up-regulation of Necl-5 is mediated by the Raf-MEK-ERK-AP-1 pathway in NIH3T3 cells transformed by an oncogenic Ki-Ras (V12-Ki-Ras) [8,15,17]. We therefore examined here whether up-regulated Necl-5 contributes to the loss of contact inhibition of cell movement and proliferation in transformed NIH3T3 cells.

Materials and methods

Cell culture. NIH3T3 cells were obtained from the American Type Culture Collection. V12-Ki-Ras-NIH3T3 cells were prepared as previously described [18]. NIH3T3 and V12-Ki-Ras-NIH3T3 cells were maintained in DME supplemented with 10% calf serum. For cultures at a low or high density, V12-Ki-Ras-NIH3T3 cells were seeded at a density of 1×10^4 or 8×10^4 cells/cm^2, respectively, and cultured for 24 h. Knockdown of Necl-5 using the small interfering RNA (siRNA) vector pBS-H1-Necl-5, and the control vector pBS-H1-control, was performed as described previously [16]. The pBS-H1 vector was a gift from Dr. Shibuya (Tokyo Medical and Dental University, Tokyo, Japan).

Antibodies and reagents. A rat monoclonal antibody (mAb) against the extracellular region of Necl-5 (1A8-8, mAb-i) was prepared as previously described [15]. A rabbit polyclonal antibody (pAb) against the extracellular region of Necl-5 was used for Western blotting. A rabbit anti-nectin-1 pAb and rabbit anti-nectin-3α pAb were prepared as described [19,20]. A mouse anti-actin mAb and mouse anti-N-cadherin mAb were purchased from Chemicon and BD Biosciences, respectively. Horseradish peroxidase-conjugated and fluorophore-conjugated secondary Abs were purchased from Amersham Biosciences and Chemicon. The fusion proteins containing the extracellular regions of nectin-1 and -3 fused to the Fc portion of human IgG (Nef-1 and Nef-3, respectively) were prepared as previously described [21] and cross-linked by a rabbit anti-human IgG Fc pAb (Jackson Immuno Research) before use.

Immunofluorescence microscopy. Cells were fixed with 1% formaldehyde in PBS for 15 min, permeabilized with 100% methanol at −20 °C for 10 min, incubated with 1% BSA in PBS, and then incubated with 20% BlockAce in PBS. The cells, which were pre-cultured with the anti-Necl-5 mAb-i, were washed with a stripping buffer (0.2 M acetic acid and 0.5 M NaCl) before fixation. Immunostaining was performed as described [7].

Boyden chamber assay. The Boyden chamber assay was performed as previously described [7] with some modifications. V12-Ki-Ras-NIH3T3 cells were seeded at a density of 4×10^4 cells/inset. The cells were incubated at 37 °C for 9 h in the presence of 10% serum. Migrated cells, which expressed EGFP-tub, were counted by fluorescence microscopic examination.

Other procedures. Quantification of cell surface proteins and endocytosis of Necl-5, by a biotinylation-based method, were performed as previously described [22]. The endocytosis assay was performed at 18 °C to inhibit recycling. The DNA synthesis assay was performed using BrdU labeling and detection kit I (Roche), according to manufacturer's protocols. SDS–PAGE was performed as previously described [23]. Protein concentrations were determined using bovine serum albumin as a reference protein, as previously described [24].

Results

We have previously shown that Necl-5 is up-regulated in V12-Ki-Ras-NIH3T3 cells [15]. We first examined, by

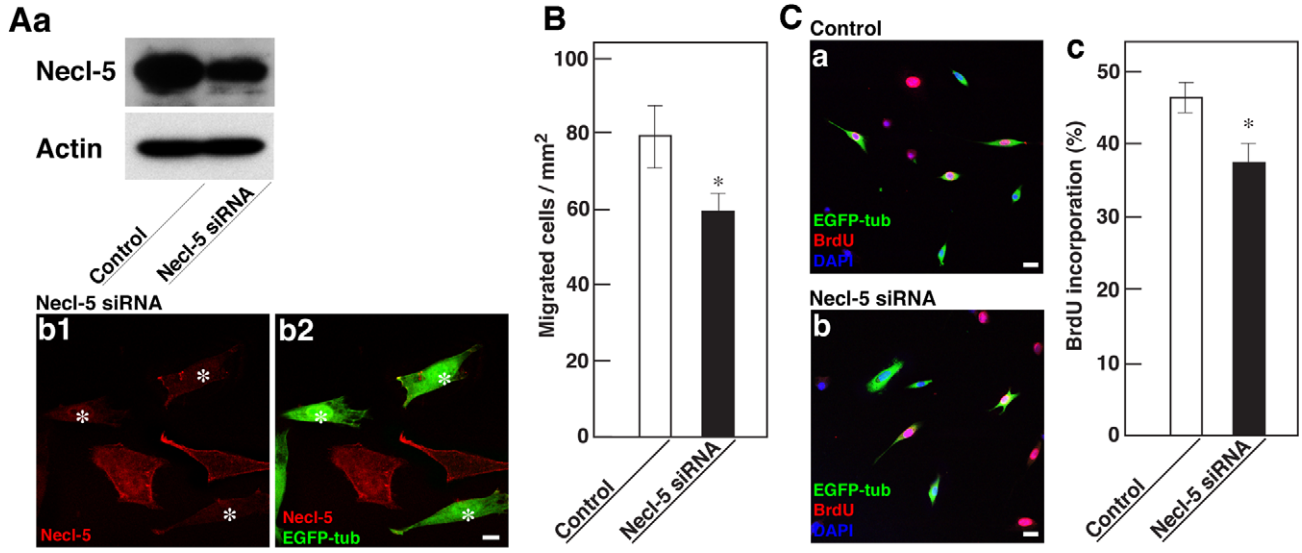

Fig. 1. Reduction of movement and DNA synthesis in V12-Ki-Ras-NIH3T3 cells by knockdown of Necl-5. V12-Ki-Ras-NIH3T3 cells were transfected either with the siRNA vector against Necl-5 and pEGFP-tub vector or with the control siRNA vector and pEGFP-tub vector, and cultured at a low cell density for 48 h. The transfection efficiency was about 20%. (A) Confirmation of knockdown of Necl-5 (a) Western blotting. EGFP-tub-positive cells were sorted by FACS® and the lysate of the sorted cells was subjected to SDS–PAGE (10% polyacrylamide gel), followed by Western blotting with the anti-Necl-5 and the anti-actin mAbs. (b) Immunofluorescence images. Cells were single stained with the anti-Necl-5 mAb. (b1) Necl-5; (b2) Necl-5 and EGFP-tub. Asterisks, Necl-5-knockdown cells; bar, 10 μm. (B) Measurement of cell movement by the Boyden chamber assay. Cells were seeded in a culture insert and cultured for 9 h. EGFP-tub-positive cells that had migrated were counted $^*P < 0.003$. (C) Measurement of DNA synthesis by the BrdU incorporation assay. Cells were re-plated at a low cell density, cultured for 18 h, and incubated with BrdU for 2 h. Cells were double stained with the anti-BrdU mAb and DAPI. (a) Control; (b) Necl-5 siRNA; (c) quantification of BrdU-positive cells. Bars, 20 μm; $^*P < 0.003$. The results shown in (A) are representative of three independent experiments, and the results shown in (B,C) are means ± standard error of the three independent experiments.

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