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Spatiotemporally-regulated interaction between β1 integrin and ErbB4 that is involved in fibronectin-dependent cell migration

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Integrins are widely expressed cell surface molecules that mediate cell attachment to extracellular matrix (ECM) proteins. They also interact with molecules on their own membranes, and these *cis*-interactions play a crucial role in integrin-dependent cellular responses. We herein analysed what molecules interact with β1 integrin during biological events induced by cell attachment to different ECM proteins, using a recently established reaction, the enzyme-mediated activation of radical sources (EMARS). The interactions between β1 integrin and receptor tyrosine kinases including EGFR and ErbB4 reached a peak at 2 h after seeding HeLa S3 cells onto the ECM proteins. The peak of phosphorylation of ErbB4 (at 2 h after seeding the cells onto fibronectin) coincided with the peak of the interaction with β1 integrin, while that of EGFR (at 1 day) did not. Accompanying with these findings, suppression of cell migration by a pharmacological inhibitor of the ErbB family receptors, PD168393 and an anti-ErbB4 neutralizing antibody, 12D8 was observed at 2 h after seeding. Taken together, it is deduced that interactions between β1 integrin and ErbB4 occur in a spatiotemporally-regulated manner, and such interaction contributes to the integrin-dependent cell migration.

Keywords: β1 integrin/cell migration/fibronectin/ErbB4/EMARS.

Abbreviations: ECM, extracellular matrix; EGF, epidermal growth factor; EGFR, epidermal growth factor receptor; EMARS, enzyme-mediated activation of radical sources; HRP, horse radish peroxidase; NRG1, neuregulin 1; RTK, receptor tyrosine kinase.

Cell adhesion and migration are basic biological events to form tissues and organs. These events are mediated by the contact between cell surface and ECM. The main cell surface adhesion molecules are the integrin family receptors (*1*) and they bind to related ECM proteins, such as fibronectin, collagen and laminin (*2*). The integrin family members generally exist as heterodimer of α and β subunits (*1*). There are several kinds of α and β subunits in mammalian cells, and both subunits are membrane-integrated proteins. The distinct combinations of two subunits determine the ligand specificity and bring about specific cellular responses (*1*). β1 integrin is the most common β subunit, and plays pivotal roles in cell adhesion and migration, collaborating with various α subunits. In addition, β1 integrin has an effect on cell survival and proliferation and these effects vary dependent on cell types and developmental stages (*3*, *4*).

In addition to the communication between integrins and the ECM proteins, recent studies have shown that *cis*-interaction, or lateral association between integrins and other cell surface molecules yields intracellular signals leading to the integrin-mediated cellular responses (*5*). For example, the interactions between EGFR and integrins promote cell proliferation (*6*–*8*). Molecules embedded in the plasma membrane dynamically move about and repeatedly associate and dissociate with each other to form molecular clusters, called lipid rafts or membrane microdomains (*9*). Since integrins are known to be localized at the lipid rafts (*10*), the coupling between the *trans*- (integrin-ECM proteins) and the *cis*- (integrin-RTKs) interactions is considered to take place in the lipid rafts. Such coupling of the interactions involving integrins, however, has not been studied under physiological states.

Although integrins are constitutively expressed on the cell surface, their molecular functions change over time, accompanied by the transition of biological events from cell adhesion to migration to proliferation and so on (*1*). Therefore, in order to understand the signaling pathways leading to the integrin-dependent biological responses, time factor must be taken into account. Following cell attachment to ECM, tight adhesion, migration and mitosis take place within minutes, hours and days, respectively.

In this study, we have investigated the changes in coclustering molecules with β1 integrin in the same membrane microdomain during biological events induced by cell contact to the three kinds of ECM proteins, fibronectin, type 1 collagen or laminin, using a recently established reaction, called the

enzyme-mediated activation of radical sources (EMARS), to label molecules within ~200–300 nm from the probed molecule on which HRP is set (*11*). We have also explored the link between one of the coclustered molecule, ErbB4 and the coincident biological event, cell migration.

Materials and Methods

Cell culture

HeLa S3 and MDA-MB231 cells were cultured in RPMI 1640 and DMEM medium, respectively, supplemented with 10% foetal bovine serum (FBS) at 37°C under humidified air containing 5% CO_2. Before the EMARS reaction, confocal laser scan microscopy observation, or cell adhesion assay, the cells were cultured with serum-free ASF104 medium (Ajinomoto), overnight. In the case of cell migration assay, the cells were cultured with RPMI 1640 containing 1% BSA, overnight. Mouse hybridoma TS2/16 cells were cultured in ASF104 medium.

Preparation of HRP-conjugated monovalent TS2/16 antibody

An anti-β1 integrin monoclonal antibody, TS2/16, was purified from the conditioned media of the hybridoma using a protein G-Sepharose (GE Healthcare). The purified TS2/16 antibody was partially reduced and bound to HRP using a peroxidase labeling kit SH (Dojindo) (Supplementary Fig. S1A). Anti-HRP antibody (Jackson Immunoresearch) was labelled with FITC (Sigma), and purified using a G-50 Sepharose (GE Healthcare) spin column. The prepared HRP-conjugated monovalent TS2/16 antibody was validated as follows. HeLaS3 cells were incubated with the antibody and subsequently with two kinds of second antibodies, Alexa Fluor 488-conjugated anti-mouse IgG (Invitrogen) and FITC-conjugated anti-HRP antibody, separately, and then analysed by flowcytometry with a FACScan equipped with CellQuest (BD Biosciences) (Supplementary Fig. S1B).

The EMARS reaction

Culture plates were precoated with 10 μg/ml of fibronectin (Sigma), type 1 collagen (Sigma) or laminin (BD Biosciences) in PBS at 4°C overnight. HeLaS3 and MDA-MB231 cells were pretreated with serum-free ASF104 medium overnight before use. The pretreated cells were seeded onto an ECM protein-precoated plate, and cultured with ASF104 medium at 37°C for 15 min, 2 h and 1day. The cells were washed once with ASF104 medium, and then treated with 10 μg/ml of HRP-conjugated monovalent TS2/16 antibody or non-labelled monovalent TS2/16 antibody in ASF104 medium at 37°C for 20 min. After washing, the cells were subsequently incubated with 0.1 mM arylazide-fluorescein synthesized in our hands (details will be published elsewhere) in PBS at 37°C for 20 min in dark to proceed the EMARS reaction. The resulting cells were removed into a plastic tube with 100 mM Tris–HCl (pH 7.4), and then crushed by passing through a 21 G syringe needle. The microsome fractions were recovered by the method as described previously (*11*). The microsome pellets were dissolved in the lysis buffer [20 mM Tris–HCl (pH 7.4), 150 mM NaCl, 5 mM EDTA, 1% NP-40, 10% glycerol] and analysed on SDS–PAGE or RTKs antibody array.

Analysis of the EMARS products

We analysed the EMARS products by SDS–PAGE and Human Receptor Tyrosine Kinase Antibody Array (ARY001; R&D systems). Following the EMARS reaction, samples (10 μg total protein) were subjected to SDS–PAGE (10% gel, under a non-reducing condition), and detected using a LAS-4000 fluorescence image analyser (Fuji film) equipped with blue light and Y515-Di filter under fluorescence mode. The method for the RTKs antibody array was according to the manufacture's manual except for detection. Ten microgram total protein of the EMARS product was applied to antibody array. To detect fluorescein-labelled molecules, an anti-fluorescein antibody (Rockland), conjugated with HRP by our hands using a peroxidase labelling kit NH2 (Dojindo), was used. The detection of tyrosine phosphorylation in each RTK was performed using an HRP-conjugated anti-phosphotyrosine antibody included in the antibody array set.

Flowcytometry analysis

HeLaS3 and MDA-MB231 cells cultured on fibronectin-precoated plates for 15 min, 2 h and 1 day were harvested by the same way as the EMARS reaction. The cells were collected into 1.5 ml microtubes, and washed with PBS at 4°C. The cells were stained with HRP-conjugated monovalent TS2/16 antibody (20 μg/ml) or anti-human ErbB4 (12D8) antibody (17.1 μg/ml) at 4°C for 30 min., and then with 10 μg/ml of Alexa Fluor 488-conjugated anti-mouse IgG (Invitrogen) at 4°C for 30 min. The treated cells were analysed by flowcytometry with a FACScan equipped with CellQuest.

Cross-linking experiment

HeLaS3 cells cultured on fibronectin-precoated plates for 15 min, 2 h and 1 day were harvested by the same way as the EMARS reaction. The cells were subsequently treated with 0.3 mg/ml bis (sulphosuccinimidyl) suberate (BS^3, Thermo Co.) in PBS at room temperature for 15 min. After washing with PBS, the microsome fractions were recovered and solubilized with the lysis buffer by the same way as the EMARS reaction. Samples were immunoprecipitated with 2 μg TS2/16 antibody and Ab-capture Protein A-Sepharose (Protenova). Immunoprecipitated samples were subjected to SDS–PAGE (6% gel, under a reducing condition), blotted onto a PVDF membrane (Millipore), and then treated with anti-human ErbB4 (12D8) monoclonal antibody (3.4 μg/ml) purified from conditioned media of the hybridoma, at room temperature for 1 h. The membranes were subsequently treated with TrueBlot anti-mouse IgG-HRP (eBioscience; 1 : 2,000) at room temperature for 1 h and developed with an Immobilon Western Chemiluminescent HRP Substrate (Millipore).

Confocal microscopy

HeLaS3 cells were seeded onto confocal plates (Matunami Glass) precoated with 10 μg/ml fibronectin and incubated at 37°C for 15 min, 2 h and 1 day. The cultured cells were fixed with 3% formaldehyde in PBS at room temperature for 15 min, and then treated with anti-human ErbB4 (12D8) antibody (34.2 μg/ml) at room temperature for 30 min. The cells were subsequently treated with 5 μg/ml of Alexa Fluor 635-conjugated anti-mouse IgG (Invitrogen) at room temperature for 30 min. Then, Alexa Fluor 488-conjugated anti-β1 integrin antibody (1 : 200; BioLegend) was treated at room temperature for 30 min. The specimens were observed with a confocal laser scan microscopy (FLUOVIEW FV1000, OLYMPUS) including differential interference contrast (DIC) image.

Cell adhesion and migration assay

For the adhesion assay, a 12-well plate was coated with 10 μg/ml of fibronectin at 4°C overnight. HeLa S3 cells (1×10^5) were pretreated with anti-human ErbB4 (12D8) antibody (30 μg/ml), and then seeded onto the fibronectin-precoated plate. At 15 min, 2 and 5 h after seeding, the number and shape of attached cells were observed with IMT-2 phase contrast microscopy equipped with DP-12 digital camera unit (OLYMPUS).

For the migration assay, the backside of the membrane of the trans-well (BD BioCoat control insert; BD Biosciences) was precoated with 40 μg/ml of fibronectin at 4°C overnight followed by blocking with 1% BSA in PBS at 37°C for 1 h. In the experiment using a chemical inhibitor, HeLaS3 cells (2.5×10^5) were pretreated with 1 nM PD168393 (CALBIOCHEM) in DMSO or DMSO alone (for the control) at 37°C for 20 min. In the experiment using the anti-ErbB4 neutralizing antibody or anti-human EGFR neutralizing antibody (528) (CALBIOCHEM; for control experiment), HeLaS3 cells (2.5×10^5) were pretreated with anti-human ErbB4 (12D8) antibody (60 μg/ml), anti-human EGFR antibody (528) (5 μg/ml), or mouse IgG1 isotype control (R&D systems; 60 or 5 μg/ml; for the control) at 37°C for 20 min. After these treatments, the cells were treated with or without NRG1 (BD Biosciences) (10 and 100 nM) or 10 nM EGF (Higeta Syoyu; for control experiment) at 37°C for 20 min. The treated cells were removed to the precoated trans-well and cultured for 2 h. The migrated cells (move to backside) were fixed and stained with 0.3% crystal violet (Merck) in 3% formaldehyde for 30 min. The number of the stained cells was counted by taking picture of several positions with an IMT-2 phase contrast microscopy equipped with DP-12 digital camera unit. Four independent experiments were performed. One-way analysis of variance was used to test differences among more than two means for significance. Two-tailed Student *t*-test was also

performed to test differences between two means. When *P*-value was <0.05, difference was considered significant.

Results

Interaction between cell surface molecules and β1 integrin induced by cell attachment to various ECM proteins

In order to elucidate the changes in coclustering molecules with β1 integrin over time following cell attachment to various ECM proteins, the EMARS reaction was performed at 15 min, 2 h and 1 day after seeding HeLa S3 cells onto dishes precoated with fibronectin (*FN*), type 1 collagen (*COL*) and laminin (*LN*) (method is schematically shown in Fig. 1). Although arylazide-biotin used to be utilized as a labelling reagent in the original report (*11*), an improved labelling reagent, arylazide-fluorescein was employed in this study. The advantage of arylazide-fluorescein is to enable to detect the label directly using a fluorescence imager, and to reduce the non-specific labelling by endogenous enzyme(s) (details will be published elsewhere).

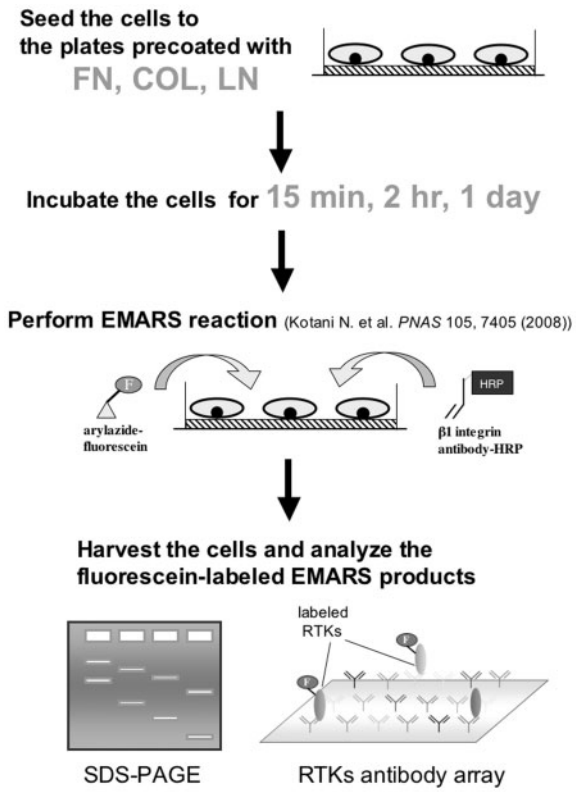

Fig. 1 Scheme of the method for analysis of the interaction with β1 integrin. Culture dishes were precoated with fibronectin (*FN*), type 1 collagen (*COL*) or laminin (*LN*). After seeding HeLa S3 cells onto the precoated dishes, the cells were incubated for 15 min, 2 h and 1 day and applied to the EMARS reaction using an HRP-conjugated monovalent anti-β1 antibody and arylazide-fluorescein as described in 'Materials and Methods' section. The HRP-conjugated monovalent anti-β1 antibody was prepared by reducing the TS2/16 antibody (Supplementary Fig. S1). The EMARS products were subsequently subjected to SDS–PAGE or RTKs antibody array as described in 'Materials and Methods' section.

First, fluorescein-labelled EMARS products as coclustered molecules with β1 integrin were detected using a LAS-4000 fluorescence imager following SDS–PAGE analysis. As a result, many fluorescent bands were seen in the samples where HRP-conjugated anti-β1 integrin antibody was used as probe (Fig. 2A). In contrast, only faint signals were detected in the samples where non-labelled β1 integrin antibody was used as negative control (Fig. 2B). These results clearly demonstrate that the fluorescein labelling depended on the EMARS reaction by HRP set on the cell surface β1 integrin.

The labelled bands were apparently similar to each other but partially different between samples cultured on different ECM proteins (Fig. 2A). For example, the labelling intensity at 15 min was stronger to weaker in

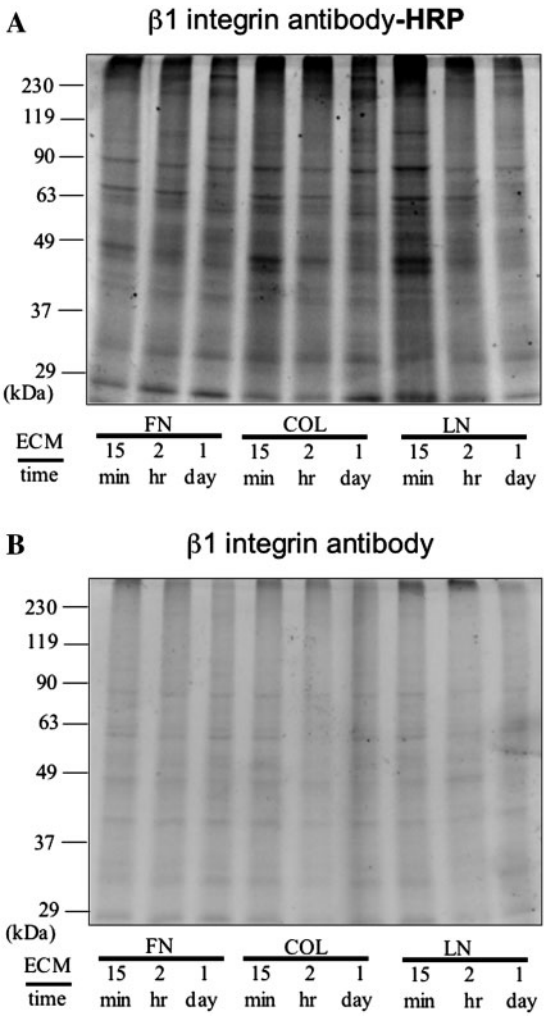

Fig. 2 Interaction between β1 integrin and whole cell surface molecules. The EMARS reaction was performed at 15 min, 2 h and 1 day after seeding of HeLa S3 cells onto dishes precoated with fibronectin (*FN*), type 1 collagen (*COL*) or laminin (*LN*), in the presence of an HRP-conjugated monovalent anti-β1 integrin antibody (A) or non-labelled β1 integrin antibody (B) as a probe and arylazide-fluorescein as a labelling reagent. Following the EMARS reaction, microsome fraction was subjected to SDS–PAGE (10% gel) and fluorescein-labelled molecules were detected using a LAS-4000 fluorescence imager as described in 'Materials and Methods' section.

the samples of laminin (*LN*), type 1 collagen (*COL*) and fibronectin (*FN*) in this order. On the other hand, the labelling intensity was highest at 15 min and gradually attenuated over time, but other labelled bands emerged later in all the ECM protein cases (Fig. 2A). For example, the two bands of 100 and 60 kDa appeared only after 1 day with regard to the fibronectin (*FN*) sample. Similar phenomenon was observed in the case of collagen (*COL*) sample. As to the laminin (*LN*) sample, the labelling intensity was very weak at 1 day while it was strongest at 15 min as mentioned above. Taken together, these results indicate that the *cis*-interaction of β1 integrin changes depending on the ligand ECM proteins and the transition of biological events over time.

Interaction between RTKs and β1 integrin induced by cell attachment to various ECM proteins

Since the interactions between EGFR and integrins have been reported (*6–8*), next we focused on the interaction between RTKs and β1 integrin, by combination of the EMARS reaction and RTKs antibody array analysis. The EMARS products of the fibronectin samples were applied to the RTKs antibody array. Labelled RTKs were identified using an anti-fluorescein antibody. As shown in Fig. 3A, many RTKs, including EGFR, ErbB4, MSPR, Tie-2, VEGFR3 and MuSK, were labelled. The labelling intensity was strongest at 2 h, and ErbB4, Tie-2, VEGFR3 and MuSK were predominantly labelled, indicating that the *cis*-interaction between β1 integrin and such RTKs undergoes most frequently at 2 h after seeding HeLa S3 cells onto fibronectin-precoated dishes. Similarly, the labelling intensity was strongest at 2 h, and EGFR, ErbB4, MSPR and Tie-2 were predominantly labelled in the collagen samples, although the signals were weaker than the fibronectin samples (Fig. 3B). In the laminin samples, EGFR, ErbB4 and Tie-2 were robustly labelled at 2 h and 1 day (Fig. 3C). In contrast to the result of the interaction of β1 integrin with whole cell surface molecules (Fig. 2A), the interaction with RTKs was found to occur more frequently at 2 h than 15 min after cell contact to the ECM proteins. Moreover, a human breast cancer cell line, MDA-MB231 was examined using the EMARS reaction under the same condition with HeLa S3 cells to investigate the issue of whether the observed interactions between β1 integrin and RTKs are common phenomena or not. The RTKs interacting with β1 integrin in MDA-MB231 cells were different from those in HeLa S3 cells. The EGF receptor and EphA2 were obviously detected in MDA-MB231 cells (Fig. 3D). In contrast to HeLa S3 cells, the interaction between β1 integrin and some RTKs, such as ErbB4 and EphA2, was stronger in 15 min culture compared to 2 h and 1 day culture. These findings indicate that the interaction between β1 integrin and RTKs is punctually regulated by the time-ordered mechanisms triggered by cell attachment to the ECM proteins and differs depending on the cell lines.

Coincidence between the cis-interaction with β1 integrin and phosphorylation of ErbB4

Since phosphorylation of RTKs is quite important for signal transduction pathways, relationship between their interaction with β1 integrin and phosphorylation was investigated. The same fibronectin samples as for the previous interaction analysis were simultaneously applied to another antibody array for detection of phosphorylation using an anti-phosphotyrosine antibody. As shown in Fig. 3E, EGFR and ErbB4 were predominantly phosphorylated. The extent of phosphorylation of EGFR gradually increased over time and was highest at 1 day after seeding the cells, not correlating with that of the interaction with β1 integrin (Fig. 3A), which underwent most frequently at 2 h and hardly occurred after 1 day. In contrast to EGFR, the phosphorylation of ErbB4 was highest at 2 h, in accordance with the interaction with β1 integrin (Fig. 3A).

To exclude the possibility that the results of the interaction with β1 integrin and phosphorylation described above reflected the expression levels of β1 integrin and ErbB4, their expression levels were confirmed by flowcytometry analysis. As shown in Fig. 3F, the expression level of β1 integrin and ErbB4 was almost constant over 15 min, 2 h and 1 day culture. This result showed that the up-regulations of the interaction with β1 integrin and phosphorylation of ErbB4 at 2 h are independent on their expression levels.

Cross-linking experiment of the interaction between β1 integrin and ErbB4

Next, we additionally performed cross-linking experiment using a chemical cross-linker to verify the interaction between β1 integrin and ErbB4. After cross-linking experiment, β1 integrin was immunoprecipitated with TS2/16 antibody. The immunoprecipitated samples from 15 min, 2 h and 1 day culture were subsequently applied to western blot analysis with anti-ErbB4 antibody. As a result, bands of cross-linked complex between β1 integrin and ErbB4 were observed at over 195 kDa (Supplementary Fig. S2). Larger bands including additional molecules were also detected in 2 h culture sample more intensely than those in 15 min and 1 day culture samples (Supplementary Fig. S2). This result supports the interaction between integrin and ErbB4.

Morphological observation of the interaction between β1 integrin and ErbB4

To verify the interaction between β1 integrin and ErbB4 morphologically in addition to the biochemical analysis, immunocytochemical observation was performed using a confocal laser scan microscopy. ErbB4 was usually located in the intracellular organelle, whereas most β1 integrin was resident in the plasma membranes (Fig. 4). At 2 h, a part of ErbB4 was mobilized to a particular portion of leading edge, where β1 integrin was colocalized. This result suggests that ErbB4 interacts with β1 integrin in a spatiotemporally regulated manner.

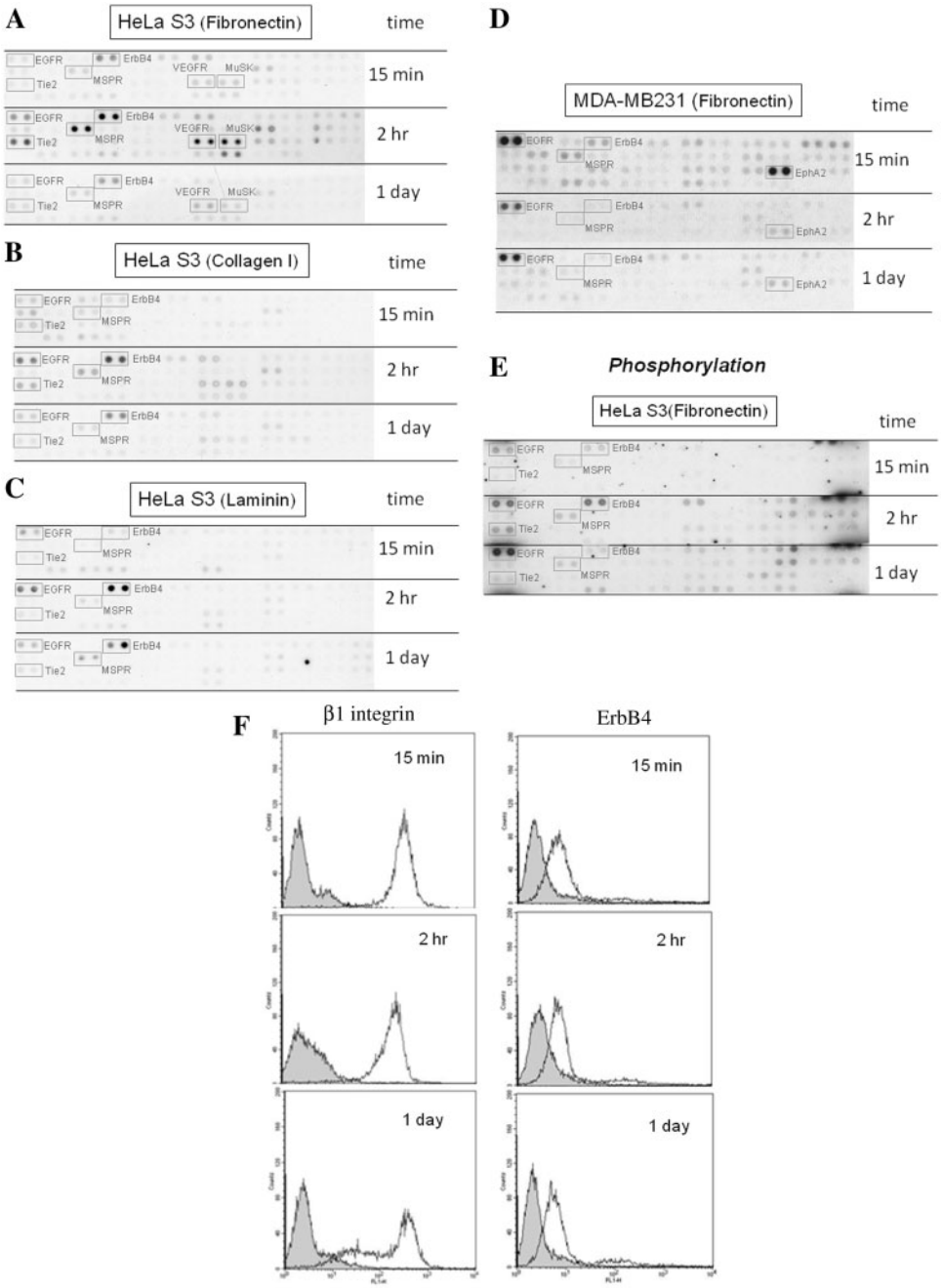

Fig. 3 Interaction with β1 integrin and phosphorylation of RTKs. (A–C) Interaction between β1 integrin and RTKs in HeLa S3 cells. The EMARS reaction was performed at 15 min, 2 h and 1 day after seeding of HeLa S3 cells onto dishes precoated with fibronectin (A), type 1 collagen (B) or laminin (C), in the presence of an HRP-conjugated monovalent anti-β1 integrin antibody as a probe and arylazide-fluorescein as a labelling reagent. (D) Interaction between β1 integrin and RTKs in MDA-MB231 cells. The EMARS reaction was performed at 15 min, 2 h and 1 day after seeding of MDA-MB231 onto dishes precoated with fibronectin, in the presence of an HRP-conjugated monovalent anti-β1 integrin antibody as a probe and arylazide-fluorescein as a labelling reagent. The EMARS products (10 μg total protein) were applied to the RTKs antibody array as described in 'Materials and Methods' section. (E) Phosphorylation of RTKs. The EMARS product as described above was applied to another antibody array. The extent of phosphorylation was analysed using an anti-phosphotyrosine antibody as described in 'Materials and Methods' section. (F) Flowcytometry analysis of β1 integrin (left column) and ErbB4 (right column) in the cells cultured on fibronectin-precoated dishes for the indicated times. Sample preparation and flowcytometry were performed as described in 'Materials and Methods' section.

ErbB4 is a regulator of the cell migration of HeLa S3 cells induced by cell attachment to fibronectin

The coincidence between the interaction with β1 integrin and phosphorylation prompted us to investigate the issue of whether ErbB4 is responsible for the integrin-dependent biological responses occurring around 2 h after cell attachment to fibronectin. To this end, effects of a pharmacological inhibitor for the ErbB family receptors, PD168393 and an anti-ErbB4 neutralizing antibody, 12D8 on cell adhesion and migration of HeLa S3 cells cultured on fibronectin-precoated dishes were examined. First, cell adhesion was tested

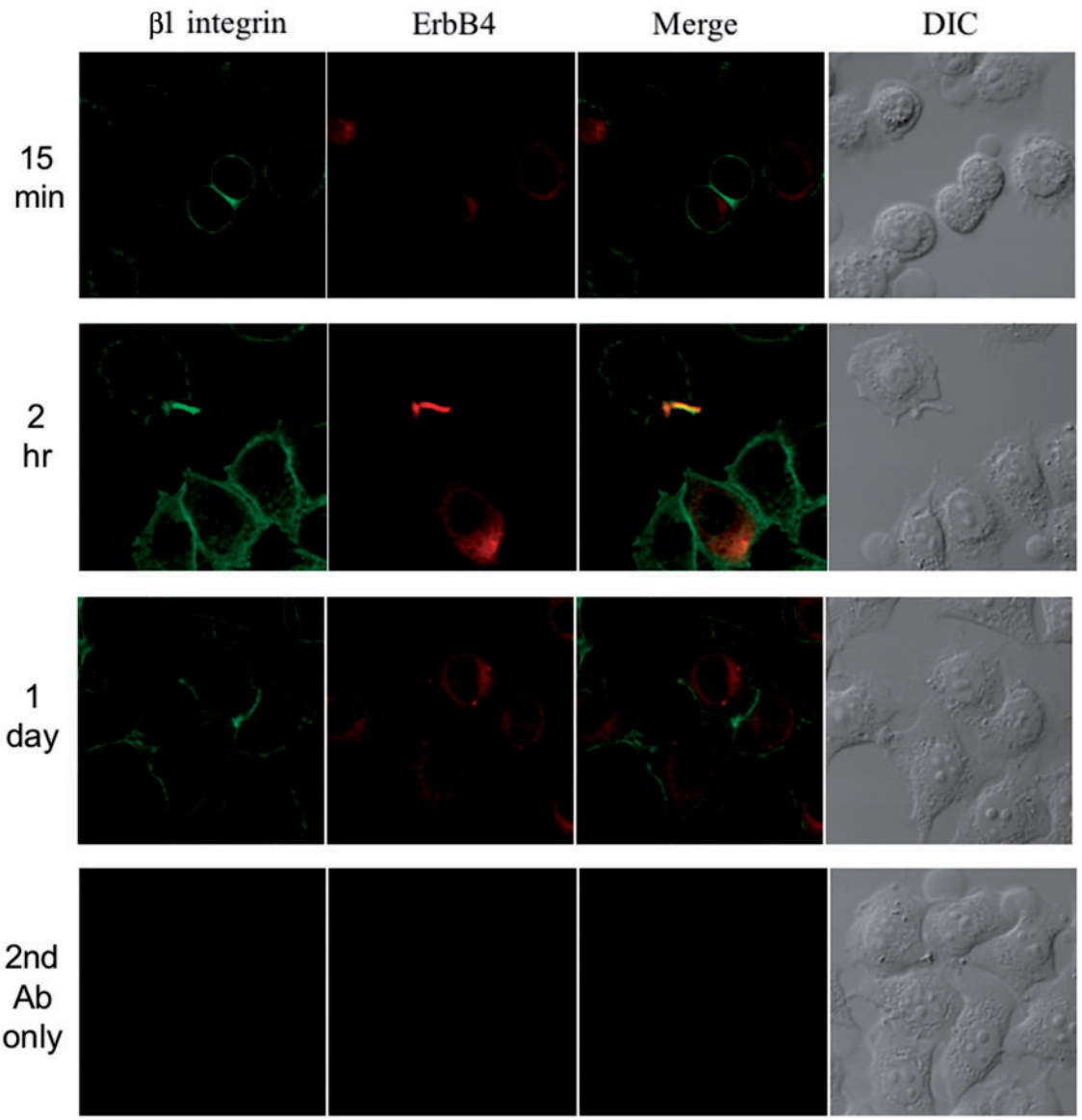

Fig. 4 Localization of β1 integrin and ErbB4 in HeLa S3 cells. HeLa S3 cells were cultured on fibronectin-precoated dishes for the indicated times, fixed with 3% formaldehyde, and stained for β1 integrin with Alexa Fluoro 488-conjugated anti-β1 integrin antibody (green), and for ErbB4 with anti-human ErbB4 antibody, 12D8 followed by Alexa Fluoro 635-conjugated anti-mouse IgG (red). As negative control, the cells were stained with Alexa Fluor 635-conjugated anti-mouse IgG alone (second Ab only). DIC, differential interference contrast.

with or without the anti-ErbB4 neutralizing antibody. Under a phase contrast microscopy, there was no significant difference in the number and shape of adherent cells until 5 h after seeding (data not shown), indicating that ErbB4 is not responsible for the cell adhesion of HeLa S3 cells on fibronectin.

Next, effects of the pharmacological inhibitor and the anti-ErbB4 neutralizing antibody on the cell migration were examined using the trans-well assay. Administration of PD168393 significantly inhibited the cell migration (Fig. 5A, the left two bars), suggesting that activation of some ErbB family receptors is required for the cell migration induced by cell attachment to fibronectin. PD168394 inhibits not only ErbB4 but also the other ErbB family receptors. Since ErbB2 and ErbB3 were not expressed on the cell surface (*11*) and not phosphorylated at any time (Fig. 3E in the present study) in HeLa S3 cells, their participation can be excluded. Even so, we are not able to specify whether ErbB4 is responsible for the cell migration because the anti-EGFR neutralizing antibody inhibited cell migration (Supplementary Fig. S2). Alternatively, effects of NRG1, which is a ligand for ErbB4 but not for EGFR, on the cell migration were examined to confirm the potentiality of the NRG1-ErbB4 pathway. When NRG1 was added in the concentration of 10 and 100 nM, the cell migration was promoted in a dose-dependent manner (Fig. 5A). Furthermore, simultaneous administration of PD168394 canceled the enhancement by NRG1 (Fig. 5A), indicating that the effect of NRG1 is mediated by ErbB4. These results indicate that activation of the ErbB4 pathway is able to promote the cell migration induced by cell attachment to fibronectin.

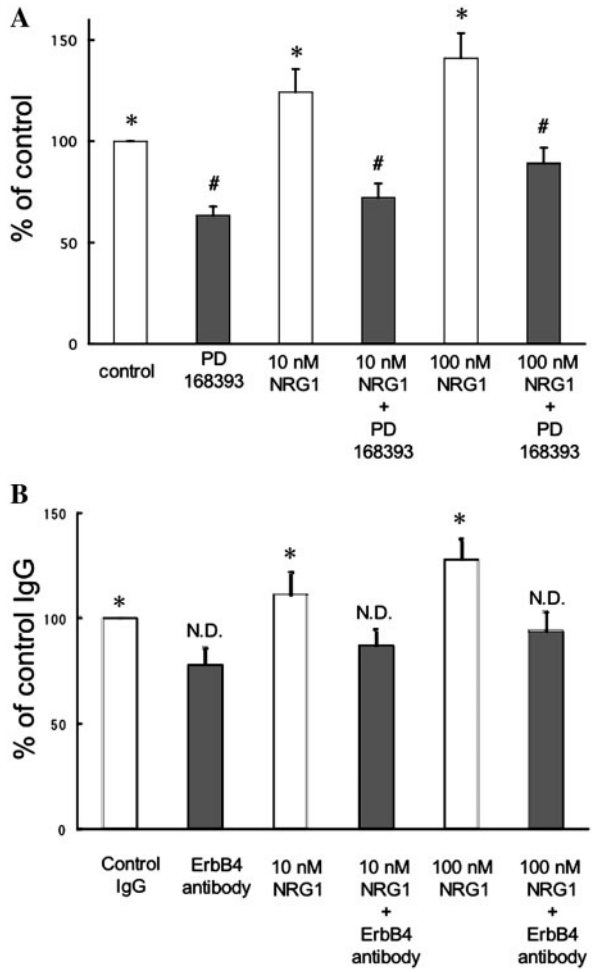

Fig. 5 Effect of a pharmacological inhibitor for ErbB family receptors and an anti-ErbB4 neutralizing antibody on the cell migration on fibronectin. Cell migration was analysed by the trans-well assay as described in 'Materials and Methods' section. (A) HeLa S3 cells were pretreated with or without PD168393 (1 nM) for 20 min and subsequently treated with or without the indicated concentration of NRG1 for 20 min. Then the treated cells were applied to a trans-well precoated with fibronectin and incubated for 2 h. After incubation, cells were fixed and the number of migrated cells was counted. Four independent experiments were performed. One-way analysis of variance test for differences among without (control), with 10 nM NRG1 and 100 nM *NRG1* in the absence (asterisk) and presence (hash) of PD168393 yielded $P = 0.000706$ and 0.00098, respectively. As to the corresponding cells treated with the same concentration of NRG1, significant differences were shown between the presence and absence of PD168393 ($P < 0.05$ in two-tailed Student's *t*-test). (B) HeLa S3 cells were pretreated with anti-ErbB4 neutralizing antibody, 12D8 or mouse IgG1 isotype control for 20 min and subsequently treated with or without the indicated concentration of NRG1 for 20 min. Then the treated cells were subjected to the trans-well assay as described in (A). Four independent experiments were performed. One-way analysis of variance test for differences among without (control), with 10 nM NRG1 and 100 nM *NRG1* in the absence of 12D8 yielded $P = 0.0034$ (asterisks). No differences were observed among the samples with 12D8 pretreatment ($P = 0.06628$; N.D.). As to the corresponding cells treated with the same concentration of NRG1, significant differences were shown between the presence and absence of 12D8 ($P < 0.05$ in two-tailed Student's *t*-test).

The anti-ErbB4 neutralizing antibody canceled the enhancement of the cell migration by NRG1 (Fig. 5B), confirming that the antibody blocks the ligand-dependent ErbB4 activation. When cells were treated with the anti-ErbB4 neutralizing antibody in the absence of exogenous NRG1, cell migration was attenuated as compared with irrelevant antibody (Control IgG) (Fig. 5B, the left two bars), indicating that the ligand-dependent ErbB4 activation is at least partially involved in the cell migration induced by cell attachment to fibronectin.

Discussion

The largest subgroup of integrin family is formed by the β1 subunit containing integrins which consist of 12 members with different ECM binding properties (*1*). More than 10 signal molecules are known to bind to the cytoplasmic tail of β1 integrin, and five of which also interact with the intracellular domain of α subunit associated with β1 integrin (*12*). These signal molecules may mediate the β1 integrin-dependent signal transduction to induce important cellular responses. β1 integrin also interact with plasma membrane-integrated molecules, such as MMP1, uPAR, EGFR, CD98 and so on (*5*). Most of these interactions have been demonstrated by immunoprecipitation or chemical cross-linking. These methods, however, require static direct binding and are unable to guarantee that such interaction occurs actually under physiological condition. Experimental evidence suggesting the cross-talk of functional molecules is insufficient until actual interaction is demonstrated in living cells. We have developed a novel and widely applicable approach that makes it possible to identify cell surface molecular clustering under physiological conditions (*11*). The important feature of this system, termed as the EMARS reaction, is that activation of cross-linking reagent arylazide-tag can be accomplished not by light but by HRP. In the current study, we demonstrated the changes in the *cis*-interactions of β1 integrin during biological events triggered by cell attachment to various ECM proteins, using the EMARS reaction.

In the previous study (*11*), we found that a wide range of RTKs interact with β1 integrin in HeLa S3 cells that highly express β1 integrin on the cell surface. In that study, intact TS2/16 anti-β1 integrin monoclonal antibody and HRP-conjugated second antibody were used for the probe of the EMARS reaction. The intact TS2/16 antibody renders β1 integrin active by itself, and cross-linking of the antibodies by the second antibody disturbs membrane environment by gathering cell surface molecules. It is assumed that non-specific activation of surrounding signal molecules of the plasma membrane brought about the interaction between many kinds of RTKs and β1 integrin. In contrast to this, in the current study an HRP-conjugated monovalent anti-β1 integrin antibody was prepared (Supplementary Fig. S1) for the probe of the EMARS reaction to prevent such non-specific activation induced by cross-linking of antibodies. Expectedly, the species of the coclustered RTKs with β1 integrin (Fig. 3) dramatically decreased as compared with the previous result (*11*). Through this contrivance, we could find specific interactions and

elucidated that different ECM proteins induce distinct β1 integrin interactions.

RTKs play a crucial role in a variety of biological events, such as cell proliferation, differentiation, and apoptosis (*13*). Our result revealed that EGFR, Tie-2 and ErbB4 predominantly interact with β1 integrin in HeLa S3 cells among the 42 RTKs tested. The interactions between these RTKs and β1 integrin have been found by immunoprecipitation in other systems (*7*, *14*, *15*). As cellular events change over time, we had anticipated that the interactions between RTKs and integrin are also dynamic. That is why we investigated the changes in the interactions over time, at 15 min, 2 h and 1 day after seeding the cells onto the ECM proteins, corresponding to the time of cell adhesion, migration and mitosis, respectively. As the result, we found that the interactions between RTKs and β1 integrin most frequently occur at 2 h after seeding the cells. Among the 42 RTKs tested, two ErbB family receptors, EGFR and ErbB4 predominantly interacted with β1 integrin. In contrast to HeLa S3 cells, the interaction between β1 integrin and some RTKs (ErbB4 and EphA2) observed in MDA-MB231 cells was stronger in 15 min culture compared to 2 h and 1 day culture. The results suggested that the time-ordered interaction is dependent on cell types. On the other hand, the peak of phosphorylation of EGFR and ErbB4 in HeLa S3 cells was at 1 day and 2 h after seeding the cell, respectively. Since the peak of the interaction with β1 integrin and the peak of phosphorylation coincided in ErbB4, we focused on its biological roles. The molecular mechanism underlying the coincidence between phosphorylation and the interaction is unknown at present. Morphological observation suggests that ErbB4 is mobilized from the intracellular organelle to the leading edge of the migrating cells, where its ligand is able to bind, at 2 h after seeding the cells (Fig. 4). The activated ErbB4 might subsequently phosphorylate surrounding signal molecules that are recruited to the integrin scaffold.

The administration of the anti-ErbB4 neutralizing antibody 12D8 had no effect on the cell adhesion, suggesting that ligand-dependent activation of ErbB4 is not required for the cell adhesion. This is in accord with the results that the extent of the interaction with β1 integrin and phosphorylation of ErbB4 at 15 min were not so strong. In contrast, the cell migration at 2 h after seeding the cells, as demonstrated by the trans-well assay, was inhibited by a pharmacological inhibitor, PD168393 and the anti-ErbB4 neutralizing antibody (Fig. 5), suggesting that the activation of ErbB4 is at least partially responsible for the cell migration undergoing ~2 h after seeding the cells. The inhibitory effect of anti-EGF receptor neutralizing antibody on the cell migration suggests that the EGF-EGF receptor pathway is also responsible for the cell migration.

In conclusion, the EMARS reaction revealed that β1 integrin interacts with many cell-surface molecules including RTKs in a temporally regulated manner. Among such interactions, the interaction with ErbB4 occurring at 2 h after seeding the cells onto fibronectin is supposed to contribute to the HeLa S3 cell migration. The *cis*-interactions on the cell surface under physiological conditions that are revealed by the EMARS reaction will provide a new insight into research on a wide range of biological events.

Supplementary Data

Supplementary Data are available at *JB* Online.

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Conflict of interest
None declared.

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