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H-Ras Is a Negative Regulator of $\alpha_3\beta_1$ Integrin Expression in ECV304 Endothelial Cells

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We have examined the role of Ras in integrin expression in ECV304 endothelial cells. Among the integrins examined in stable ECV304 transfectants expressing dominant active H-Ras (DAR-ECV), expression of $\alpha_3\beta_1$ integrin showed a prominent reduction in all the DAR-ECV clones when compared to the parental ECV304 cells. This implies that H-Ras negatively regulates the expression of $\alpha_3\beta_1$ integrin in ECV304 cells. When treated with inhibitors of the Ras downstream pathway (LY294002, PD98059, SB203580), the expression of $\alpha_3\beta_1$ integrin was up-regulated most significantly by LY294002, suggesting that among the downstream pathways of Ras, phosphatidylinositol 3-kinase is a major determinant. With the application of blocking antibody to $\alpha_3\beta_1$ integrin ($2 - 2 \times 10^4$ nM), migration of ECV304 cells was enhanced to maximal (18%) at 20 nM. These results suggest that migration of endothelial cells could be modulated by H-Ras via alteration of the expression levels of $\alpha_3\beta_1$ integrin.

Integrins are integral membrane glycoproteins composed of α and β subunits (1). They are associated through non-covalent bonds and transported to the cell surface as a complex (1). Depending on cell type, different $\alpha\beta$ complexes are assembled and expressed; this determines the specific binding of each cell type to extracellular matrix protein (ECM) ligands (1). They function as cell surface receptors for ECM (2) and also mediate cell-to-cell interactions (2, 3). Alterations to integrin-mediated cell adhesion to ECM are essential regulatory processes during development, differentiation, and cell migration (3, 4, 5). Cells regulate their interactions with ECM and neighboring cells by modifying the structure and function of the integrins. This is feasible by modulating the reactivity of the integrins through activation (inside-out signal transduction) (3, 6, 7, 8) or by alteration of the expression of integrins via mobilization of specific storage pools (1).

In the inside-out signaling, the Ras proteins play a vital role. R-ras enhanced cell adhesion to ECM in mouse myeloid cells; this increase in adhesion resulted from enhanced integrin ligand-binding activity, but not from the increased integrin expression (9). On the other hand, H-Ras, and its kinase effector, Raf-1, blocked integrin activation in CHO-K1 cells (10). This suppression was not associated with integrin phosphorylation and was independent of both mRNA transcription and protein synthesis (10). Both experiments indicated that Ras protein could alter cellular adhesion via the regulation of integrin activity.

The possibility that Ras is also involved in the regulation of integrin expression was suggested from the following experiments. The downregulation of the α_2 integrin promoter activity could be achieved by overexpression of viral H-Ras in human mammary epithelial cells, and this could be reversed by a dominant negative variant of H-Ras (11). EJ-ras-transformed NIH 3T3 fibroblasts acquired a migratory phenotype on laminin-1 surfaces, and such a phenotype was accompanied by overexpression of functional $\alpha_6\beta_1$ integrin (12). Moreover, conversion of pre-β_1 integrin into mature β_1 integrins was faster in EJ-ras-transformed cells (12).

Here, we show that constitutively active H-Ras (Leu-61) downregulates $\alpha_3\beta_1$ integrin expression in the ECV304 endothelial cells and phosphatidylinositol 3 (PI3)-kinase is a major downstream effector of Ras for this effect.

MATERIALS AND METHODS

Reagents. ECV304 cell line was kindly provided by Dr. D. T. Fearon (Wellcome Trust Immunology Unit, Univ. of Cambridge School of Clinical Medicine, Cambridge, UK). Anti-H-Ras monoclonal antibody was purchased from Santa Cruz Biotechnology, Inc.

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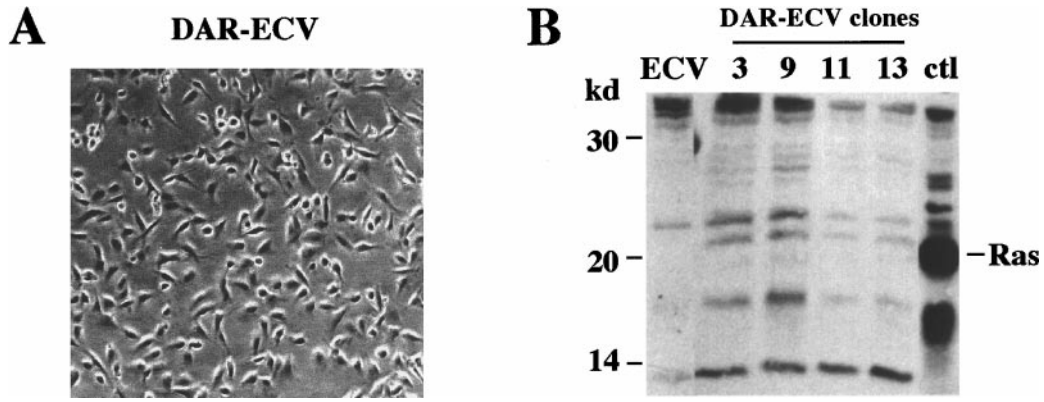

FIG. 1. Characterization of dominant, active H-ras transfected ECV304 (DAR-ECV) clones. (A) Morphology of DAR-ECV cells (Clone No. 3) has changed from the typical cobblestone apperance of parental ECV304 to fibroblast-like features. (B) For western blot analysis total cell lysate (100 μg/lane) of DAR-ECV clones (3, 9, 11, 13), ECV304 cells (ECV), and PA317 cells overexpressing H-Ras (ctl) was fractionated on a 15% sodium dodecyl sulfate polyacrylamide gel and blotted onto a nylon membrane. Ras protein was detected with anti-H-Ras antibody.

(Santa Cruz, CA). Monoclonal antibodies to human integrins $\alpha_2\beta_1$ (clone JBS2), $\alpha_3\beta_1$ (clone P1B5), $\alpha_4\beta_1$ (clone P4G9), $\alpha_5\beta_1$ (clone P1D6), and $\alpha_v\beta_3$ (clone LM609) were obtained from Chemicon (Temecula, CA) or DAKO Corp. (Carpinteria, CA). The negative antibody was MOPC21 (Cappel, West Chester, PA). The secondary antibody was DTAF-conjugated F (ab′)2 goat anti-mouse IgG (H+L) (Jackson Immunoresearch Laboratories, Inc., West Grove, PA). Ras pathway inhibitors were PD98059 (New England Biolab, Beverly, MA), LY294002 (Sigma), and SB203580 (Calbiochem, La Jolla, CA).

Transfection. pZIPrasH(Leu-61) is a constitutive expression plasmid of active H-Ras. The mutant H-ras cDNA was a kind gift from C. D. Kang (Pusan National University, Pusan, Korea). Transfection and selection of active H-Ras expressing ECV304 cells were done as previously described to establish the dominant negative H-Ras expressing DNR-ECV cell line (13). The active H-Ras expressing ECV304 cell line was named DAR-ECV.

Western blotting. ECV304 and mutant Ras expressing cells were cultured in each well of a 24-well plate in M199 with 10% fetal calf serum (FCS, GibcoBRL, Grand Island, NY). The cells were analysed by western blotting with anti-H-Ras monoclonal antibody as previously described (13).

Flow cytometry. Integrin expression pattern of ECV304 and DAR-ECV cell line was analysed by flow cytometry. Cells were stained as described in "Gene Transfer and Expression Protocols" (14) and analysed by FACSCalibur (Becton Dickinson, San Jose, CA). For the assay of inhibitory effect of Ras pathway on integrin expression, Ras pathway inhibitors were applied to ECV304 cells at different concentrations in M199 medium with 10% FCS, for 3 days. These cells were harvested and analysed for the expression pattern of $\alpha_3\beta_1$ integrin by flow cytometry.

Confocal microscopy. ECV304 cells were grown on coverslips, washed with phosphate buffered saline (PBS, GibcoBRL) and then fixed for 10 min in 4% paraformaldehyde in PBS. Fixed cells were rinsed in PBS and then permeabilized with 0.1% Triton X-100 in PBS. Normal goat serum (10% in PBS) was used to block non-specific binding. Coverslips were incubated with primary antibody at a dilution of 1:100 in PBS (monoclonal anti-$\alpha_3\beta_1$ antibody) for 1 hr at 37°C. After rinsing three times in PBS, a FITC-conjugated secondary anti-mouse IgG antibody (at a dilution of 1:100 in PBS) was applied to coverslips for 30 min at 37°C. For detection of nuclei, cells were incubated with propidium iodide (10 μg/ml). After rinsing in PBS, coverslips were mounted onto glass slides using gelvatol and observed with a confocal laser scanning microscope (Bio-Rad MRC 1024, equipped with a Zeiss Axio-plan microscope). Five to 10 focal frames, depending on cell thickness, were taken along the z axis at 0.5 μm intervals and then merged to obtain a reconstructed image.

Migration assay. Cell migration assays were performed using Nunc tissue culture inserts (TC insert, 10-mm diameter, 8-μm pores; Nalge Nunc International, Naperville, IL) containing polycarbonate membranes. Each TC insert was coated with 1% gelatin for 2 h at 37°C, then placed into a 24-well plate containing 500 μl of M199 media with 10% FCS. ECV304 cells were stained with anti-$\alpha_3\beta_1$ antibody of different concentrations in $HBSA^{++}$ solution (1 X Hank's balanced salt solution [HBSS; GibcoBRL], 0.1% bovine serum albumin [BSA, Sigma], 1.3 mM $CaCl_2$, 0.9 mM $MgCl_2$) for 30 min on ice after harvest with $HBSA^{-}$-EDTA solution (1 X HBSS, 0.1% BSA, 0.5 mM EDTA). Cells were washed with $HBSA^{++}$ solution, centrifuged and resuspended in M199 media. Cells (2×10^5) in 500 μl media were applied to the top of the gelatin coated TC insert and allowed to migrate to the underside of the TC insert for 3 hrs. Cells were fixed with 2.5% glutaraldehyde for 10 min. Non-migratory cells on the upper membrane surface were removed with a cotton swab, and the migratory cells attached to the bottom surface of the membrane were stained with 0.2% crystal violet for 20 min at room temperature. The number of migratory cells per membrane was counted as the average of eight fields with the inverted microscope using a 40 X objective lens.

RESULTS

Establishment of stable ECV304 transfectants expressing dominant active H-Ras. To examine the role of H-Ras in the expression of integrins, ECV304 endothelial cell lines expressing dominant active H-Ras constitutively (DAR-ECV) were produced by transfection with $pZIPras^{61Leu}$. The active H-ras transfectants showed transformed cell phenotypes (Fig. 1-A). They lost the typical cobblestone appearance of parental ECV304 endothelial cells. Instead, they acquired fibroblast-like features, which were more apparent when seeded into low density cultures. Moreover, they were less adherent than the parental cells. As shown in Fig. 1-B, western blotting revealed bands of H-Ras (p21) in all the transfectant clones of DAR-ECV.

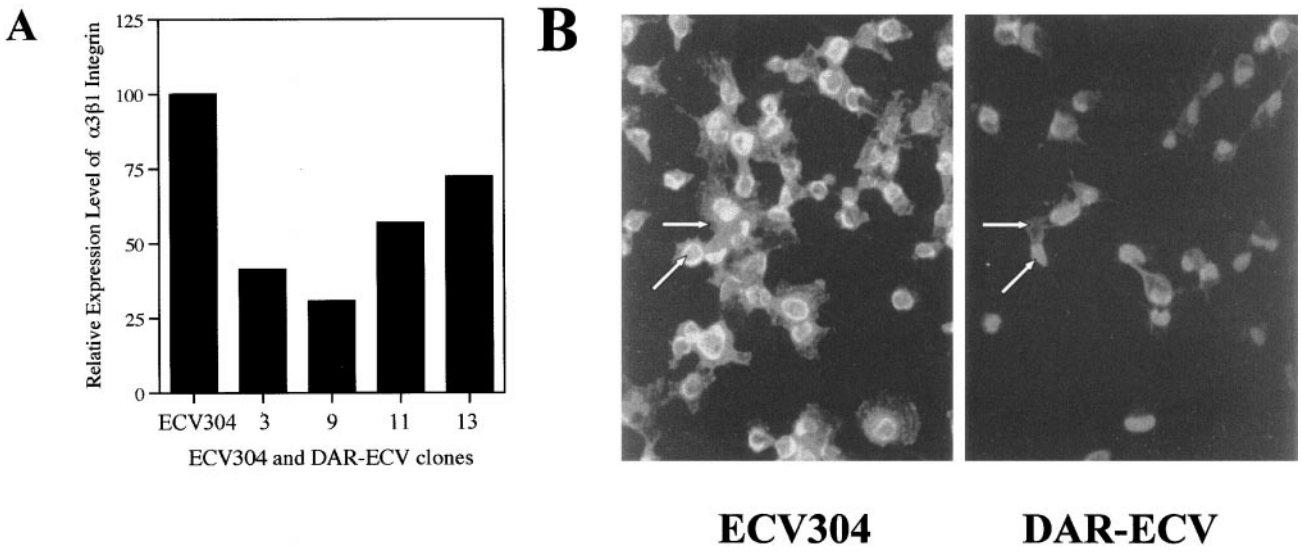

FIG. 2. Downregulation of $\alpha_3\beta_1$ integrin expression by H-Ras. (A) ECV304 cells and four DAR-ECV clones (3, 9, 11, 13) were stained with anti-$\alpha_3\beta_1$ integrin antibody followed by FITC-labeled secondary antibody and analysed by flow cytometry. Relative expression level of each clone was calculated with relative mean fluorescen level (MFL) when MFL of ECV304 is 100. (B) ECV304 cells and DAR-ECV clone 3 were grown on coverslips. Cells were fixed (4% paraformaldehyde), stained with anti-$\alpha_3\beta_1$ integrin antibody and FITC-conjugated secondary antibody, and observed with confocal microscope. Fluorescence signal for $\alpha_3\beta_1$ integrin (horizontal arrows) is stronger in the cytoplasm of ECV304 cells than in that of DAR-ECV cells. Each cell has propidium iodide stained nucleus (diagonal arrows).

Alteration of integrin expression by dominant active H-Ras. To determine whether integrin expression is altered by dominant active H-Ras, flow cytometric analysis with four different DAR-ECV clones was performed. Five integrins were selected whose expression was known to be either high ($\alpha_3\beta_1$), weak ($\alpha_v\beta_3$, $\alpha_2\beta_1$, $\alpha_5\beta_1$), or negligible ($\alpha_4\beta_1$) in endothelial cells (13). Among those integrins examined, $\alpha_3\beta_1$ integrin showed a great variation in the expression level. In all four DAR-ECV clones, $\alpha_3\beta_1$ integrin expression was consistently downregulated when compared to the parental ECV304 cells (Fig. 2-A). Confocal microscopy (Fig. 2-B) supported these results. Parental ECV304 cells exhibited strong fluorescence for $\alpha_3\beta_1$ integrin in the cytoplasm, while the staining intensity was much weaker for DAR-ECV cells (clone No 3).

Involvement of PI-3 kinase as a major regulator in integrin expression. To evaluate which downstream pathways of H-Ras are involved in the regulation of the expression of $\alpha_3\beta_1$ integrin, ECV304 cells were subjected to inhibitor analysis. Among the downstream inhibitors of the Ras pathway, LY294002 (PI-3 kinase inhibitor), SB203580 [p38 mitogen-activated protein (MAP) kinase inhibitor] and PD98059 (MAP kinase kinase inhibitor) were selected. As shown in Fig. 3, LY294002 enhanced the integrin expression most effectively in a dose-dependent manner and the maximum increment was about two-fold at 50 μM. PD98059 and SB203580 appeared to have some minor effects on the integrin expression, but less than LY294002. Moreover, the inhibitory effect of SB203580 was noticeable only at the higher concentration of 50 μM.

Role of $\alpha_3\beta_1$ integrin in the migration of ECV304 cells. To determine the biological significance of alterations in the expression level of $\alpha_3\beta_1$ integrin by Ras, we examined whether the blocking antibody (clone P1B5) to $\alpha_3\beta_1$ integrin could modulate the migratory activity of ECV304 cells. Depending on the concentra-

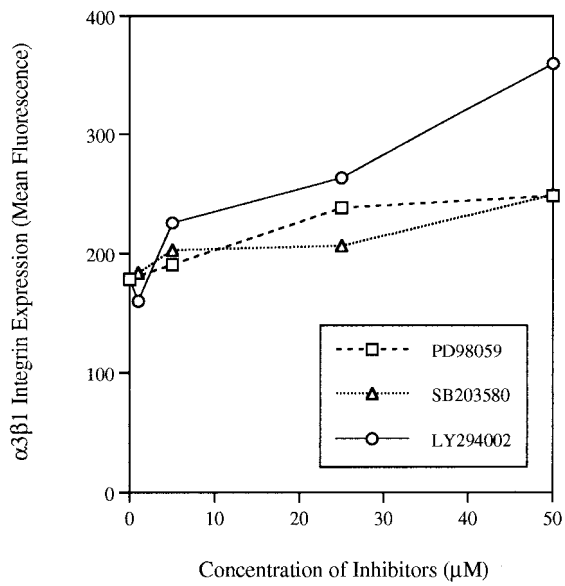

FIG. 3. Analysis of the effect of Ras pathway inhibitors on the $\alpha_3\beta_1$ integrin expression. Three different Ras pathway inhibitors (PD98059, SB203580, LY294002) were applied to ECV304 cells with the indicated concentrations in M199 medium with 10% FCS for 3 days. These cells were harvested and analysed for the expression pattern of $\alpha_3\beta_1$ integrin by flow cytometry.

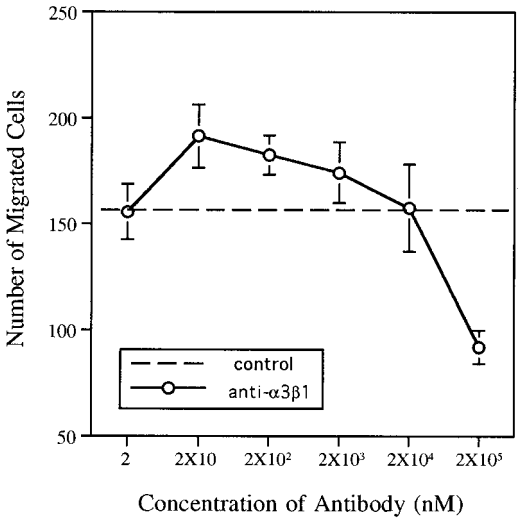

FIG. 4. Effect of anti-$\alpha_3\beta_1$ integrin antibody on migration of ECV304 cells. Cell migration assay was performed using TC insert (8 μm pore). ECV304 cells were applied to the top of 1% gelatin coated-TC insert and allowed to migrate for 3 hours in the absence (control) or presence of the indicated concentration of anti-$\alpha_3\beta_1$ integrin antibodies. Cells were fixed with 2.5% glutaraldehyde, stained with crystal violet, and counted.

tion of the blocking antibody (clone P1B5), migration of ECV304 cells was suppressed or promoted (Fig. 4). At 20 nM of this antibody, migration of ECV304 cells reached a maximum speed (118% of control cell speed), then gradually decreased with increasing antibody concentration. As the antibody concentration was greater than 20 μM, migration rate of ECV304 decreased rapidly, much less than that of control cells. This is consistent with the nature of the blocking antibody (clone P1B5). That is, at these higher concentrations binding of ECV304 cells was also severely impaired (data not shown).

DISCUSSION

Ras could alter the integrin-associated cellular physiology via two distinct pathways. One would be to modulate the integrin activity (9, 10) and the other would be to change the level of integrin expression. The latter possibility was suggested from human mammary epithelial cells overexpressing viral H-Ras (11) and EJ-ras-transformed NIH-3T3 fibroblasts (12). In this study, we presented the case that H-Ras could act as a negative regulator for expression of $\alpha_3\beta_1$ integrin in ECV304 endothelial cells.

Analysis of the effect of Ras downstream inhibitors revealed that PI3-kinase plays a major role in down-regulation of $\alpha_3\beta_1$ integrin expression in ECV304 endothelial cells. In addition, it is highly likely that Jun N-terminal kinase (JNK), but not p38 MAP kinase, is also involved in the regulation of $\alpha_3\beta_1$ integrin expression. SB203580 was initially known as a specific inhibitor of p38 MAP kinase. Recently, it has been reported that SB203580 is also inhibitory toward JNK at a higher concentration (15). In our data, SB203580 was effective in increasing $\alpha_3\beta_1$ integrin expression only at the higher concentration, suggesting that JNK is somehow related to $\alpha_3\beta_1$ integrin expression. In human osteosarcoma cells, overexpression of N-myc, one of the substrates of the extra-cellular signal-regulated kinase 1/2 (Erk1/2) (16, 17), downregulates $\alpha_3\beta_1$ integrin expression (18), and in human neuroblastoma cells, N-myc also downregulates α_2, α_3, and β_1 integrin subunits (19). However, in ECV304 cells, inhibition of Erk1/2 did not result in a significant change in $\alpha_3\beta_1$ integrin expression. These results suggest that major upstream pathways of $\alpha_3\beta_1$ integrin expression could be variable, depending on the cell types.

Both LY294002 and wortmannin are equally accepted and used as inhibitors of PI3-kinase (20–23). To our surprise, LY294002 had a dramatic effect on $\alpha_3\beta_1$ integrin expression, whereas wortmannin was ineffective (data not shown) in our experiments. A differential effect of LY294002 and wortmannin was also reported for nitric oxide production in murine macrophages (24). At present, it is not clear whether LY294002 and wortmannin have different specificity for PI3-kinase subtypes; whether the direct target of LY294002 in ECV304 cells is not PI3-kinase, but a hitherto unidentified enzyme (24).

The mechanism by which PI3-kinase could exert its effect on $\alpha_3\beta_1$ integrin expression remains unanswered. However, it is apparent that PI3-kinase is closely associated with integrin signaling. PI3-kinase could act downstream as a mediator of integrin signaling (25, 26) or upstream to activate integrin-mediated cellular events (22). In the latter case, PI3-kinase activity would lower or enhance cellular adhesion and mobilization depending on the cell types (20, 27). Most of theses cases are related to the transient activation of PI3-kinase. To date, the long-term inhibitory effect of PI3-kinase activity by wortmannin and LY294002 has not been comprehensively evaluated. In this context, our data are of some importance, suggesting that the long-term inhibition or stimulation of PI3-kinase may disrupt actin cytoskeleton structures resulting in the alteration of integrin expression.

The cell migration analysis revealed that with appropriate concentrations of blocking antibody to $\alpha_3\beta_1$ integrin, ECV304 cells migrated faster than the untreated control cells. There are several possibilities. Firstly, on ECV304 cells there might be more $\alpha_3\beta_1$ integrin molecules than the appropriate number of integrins for the usual migratory activity. This would explain why ECV304 cells are more adhesive and migrate much slower than HUVEC (13). Secondly, it is likely that the substratum adhesiveness of $\alpha_3\beta_1$ integrin is very high, and the blocking antibody might

attenuate this adhesive force and increase the cell migration. The complex process of migration can be perceived usually as a dynamic arrangement of a number of distinctive events, including membrane protrusion, formation of stable contacts between the cell and ECM, cytoskeletal contraction, cell body translocation and release of cell-substratum adhesion at the rear of the cell (28–31). Rear retraction rates usually limit cell speed at intermediate and high adhesiveness, but not at low adhesiveness (32). Our result is consistent with the notion that the blocking antibody to $\alpha_3\beta_1$ integrin regulates rear retraction during migration of ECV304 cells. That is, at the appropriate concentration range the antibody could relieve a restraint in the rear retraction process and increase the cell migration rate. DAR-ECV cells have lower $\alpha_3\beta_1$ integrin levels and migrated faster than the parental ECV304 cells (data not shown). In DAR-ECV cells, both the lower amount of integrin and decreased integrin activity by H-ras (10) presumably underlie the more efficient rear retraction than for the parental ECV304 cells during cellular migration.

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