TGF-β1 (transforming growth factor-β1)-mediated adhesion of gastric carcinoma cells involves a decrease in Ras/ERKs (extracellular-signal-regulated kinases) cascade activity dependent on c-Src activity

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Signalling by integrin-mediated cell anchorage to extracellular matrix proteins is co-operative with other receptor-mediated signalling pathways to regulate cell adhesion, spreading, proliferation, survival, migration, differentiation and gene expression. It was observed that an anchorage-independent gastric carcinoma cell line (SNU16) became adherent on TGF-β1 (transforming growth factor β1) treatment. To understand how a signal cross-talk between integrin and TGF-β1 pathways forms the basis for TGF-β1 effects, cell adhesion and signalling activities were studied using an adherent subline (SNU16Ad, an adherent variant cell line derived from SNU16) derived from the SNU16 cells. SNU16 and SNU16Ad cells, but not integrin α5-expressing SNU16 cells, showed an increase in adhesion on extracellular matrix proteins after TGF-β1 treatment. This increase was shown to be mediated by an integrin α3 subunit, which was up-regulated in adherent SNU16Ad cells and in TGF-β1-treated SNU16 cells, compared with the parental SNU16 cells. After TGF-β1 treatment of SNU16Ad cells on fibronectin, Tyr-416 phosphorylation of c-Src was increased, but Ras-GTP loading and ERK1/ERK2 (extracellular-signal-regulated kinases 1 and 2) activity were decreased, which showed a dependence on c-Src family kinase activity. Studies on adhesion and signalling activities using pharmacological inhibitors or by transient-transfection approaches showed that inhibition of ERK1/ERK2 activity increased TGF-β1-mediated cell adhesion slightly, but not the basal cell adhesion significantly, and that c-Src family kinase activity and decrease in Ras/ERKs cascade activity were required for the TGF-β1 effects. Altogether, the present study indicates that TGF-β1 treatment causes anchorage-independent gastric carcinoma cells to adhere by an increase in integrin α3 level and a c-Src family kinase activity-dependent decrease in Ras/ERKs cascade activity.

Key words: cell adhesion, c-Src, extracellular-signal-regulated kinases 1 and 2 (ERK1/ERK2), gastric carcinoma, integrin, transforming growth factor β1 (TGF-β1).

INTRODUCTION

Non-transformed epithelial cells grow in an anchorage-dependent manner by cell-adhesion-receptor-mediated cell interactions with their surrounding ECM (extracellular matrix) proteins. On the other hand, transformed cancer cells can often have an ability to grow indefinitely and in an anchorage-independent manner. This anchorage independence is believed to occur by constitutive activations of intracellular signalling molecules downstream of cell-adhesion receptors. Integrins are a family of cell-adhesion receptors, i.e. heterodimeric cell-surface receptors, consisting of α and β subunits. So far, about two dozen combinations of 18 α and 8 β subunits are known to assemble in mammalian cells. Integrins are known to regulate diverse biological functions of cells, including cell adhesion, spreading, proliferation, migration, survival and gene transcription [1–3]; they transduce signals from outside of a cell to the intracellular cytoplasm and nucleus (outside-in signalling), in addition to inside-out signalling [2–5]. Integrins can transduce signals directly to downstream intracellular signalling molecules or collaborate (indirectly) with other receptor-mediated signalling pathways, including growth factor receptors and G-protein-coupled receptors [2,6–9]. Cell adhesion and spreading are also regulated by both integrin-mediated direct signalling and collaborative (indirect) signalling, and is important for the response of cells to extracellular stimuli. In many previous studies on cell adhesion and spreading, it was reported that cell spreading involves integrin β1, activated R-Ras, Rac1, PI3K (phosphoinositide 3-kinase) and protein kinase Cε [10], p190RhoGAP [11] or SHIP (SH2-containing inositol 5′-phosphatase) [12,13]. FAK (focal adhesion kinase) activation also has been shown to contribute to cell adhesion and spreading in numerous studies [14].

TGF-β1 (transforming growth factor β1) is a multifunctional cytokine that inhibits epithelial-cell growth. It triggers intracellular signal transduction involving SMAD proteins to regulate numerous developmental and homoeostatic processes via regulation of gene induction [15–17]. Previously, TGF-β1-mediated, but SMAD-independent, signalling pathways have also been evidenced in many model systems [18–20]. For example, TGF-β1-mediated activation of p38 MAPK (mitogen-activated protein kinase) and JNKs (c-Jun N-terminal kinases) resulted in increased expression levels of ECM proteins such as collagen and fibronectin respectively [21,22]. Similarly, in diverse model systems, expression profiles of integrins are susceptible to regulation by TGF-β1 [23,24], and integrin-mediated signalling also regulates TGF-β1 expression levels [25]. Similar to growth factor

Abbreviations used: ECM, extracellular matrix; ERK, extracellular-signal-regulated kinase; GST, glutathione S-transferase; MAPK, mitogen-activated protein kinase; MEK, MAPK/ERK kinase; NRK, normal rat kidney; PI3K, phosphoinositide 3-kinase; RBD, Ras-binding domain; TGF-β1, transforming growth factor β1.

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receptors or G-protein-coupled receptors, the TGF-β1-receptor-mediated signalling pathway is also co-operative with integrin signalling. Recently, it was reported that TGF-β1 treatment of mammary epithelial cells resulted in epithelial-mesenchymal transdifferentiation and these effects were dependent on both functional integrin β1 activity and p38 MAPK activity [26].

We were interested in understanding how a cross-talk between integrin- and TGF-β1-mediated pathways modulates cellular functions. We found that anchorage-independent SNU16 gastric carcinoma cells and its adherent variant SNU16Ad showed an increase in cell adhesion after TGF-β1 treatment. Using these cell lines, we tried to understand how SNU16 and SNU16Ad cells become adherent after TGF-β1 treatment with respect to the effects of TGF-β1 treatment on signal transduction and cell adhesion. The present study reveals that TGF-β1-mediated adhesion of SNU16Ad cells involves an increased level of integrin α3, and an inverse relationship between c-Src and ERK1/ERK2 (extracellular-signal-regulated kinases 1 and 2) activities dependent on c-Src activity.

MATERIALS AND METHODS

Cells

A Korean derived gastric cancer cell line (SNU16) was purchased from Korean Cell Line Bank (Seoul, South Korea), and a subline enriched with adherent cells (SNU16Ad) was obtained from subsequent cultures by collecting adherent cells among mostly anchorage-independent cells of SNU16. SNU16 cells were stably transfected to overexpress human integrin α5 subunit by LIPOFECTAMINE™ Plus-mediated methods, and were selected by G418 drug and immunobead (Dynabeads M-450; Dynal, Oslo, Norway) using a monoclonal anti-human α5 (P1D6) antibody (SNU16α5). Cells were grown at 37 °C and in 5 % CO_2 in RPMI 60 culture media, containing 10 % (v/v) fetal bovine serum (SNU16 and SNU16Ad), and 0.2 mg/ml G418 (SNU16α5).

Cell lysate preparation and Western blots

SNU16 and SNU16α5 cells in suspension or trypsinized SNU16Ad cells were incubated in suspension in serum-free media plus 1 % (w/v) BSA for 1 h before keeping in suspension or replating on indicated ECM proteins (15 μg/ml fibronectin or laminin I; Chemicon, Temecula, CA, U.S.A.). Pharmacological inhibitors were pretreated 30 min before replating. TGF-β1 (1 ng/ml; Chemicon) was added directly to the media and the treatment lasted for 20 h. Cell lysates were prepared as described previously [27,28]. The lysates were used in Western blots using anti-ERK1/ERK2, phospho-ERK1/ERK2, phospho-Y^{416}Src (Cell Signaling Technology, Beverly, MA, U.S.A.), c-Src (New England Biolabs, Beverly, MA, U.S.A.), Ras, GST (glutathione S-transferase; BD Transduction Laboratories, San Jose, CA, U.S.A.) and anti-α3 (Santa Cruz Biotechnology, Santa Cruz, CA, U.S.A.). When the same nitrocellulose membrane was reprobed with another primary antibody, the membrane was stripped by incubating in a stripping buffer (62.5 mM Tris, pH 6.8/2 % SDS/100 mM 2-mercaptoethanol) at 65 °C for 30 min, washed for 1 h (3 × 20 min) with TBST (Tris-buffered saline with 0.05 % Tween 20), reblocked with TBST containing 1 % BSA and 2 % (w/v) non-fat milk proteins and then reprobed with another primary antibody.

Flow cytometry

Flow-cytometric measurements of integrin subtypes on cells were performed as described previously [27]. To study the TGF-β1 effects on integrin expression levels, SNU16 cells in serum-free media were treated with TGF-β1 (1 ng/ml) for 0, 12, 24 or 36 h before harvesting for the measurements. The raw data were analysed by using a software program (WinMDI version 2.7; Scripps Institute, San Diego, CA, U.S.A.).

Phase-contrast imaging

Cells replated on the indicated substrates and treated with 1 ng/ml TGF-β1 for 20 h were imaged by using a phase-contrast microscope.

Cell-adhesion assay

SNU16 and SNU16α5 cells in suspension and trypsinized SNU16Ad cells were maintained in suspension for 1 h and, in some cases, pretreated with inhibitors as described above, and replated on 96-well plates pre-coated with the indicated ECM substrates. Cells were seeded at 5×10^4/well of a 96-well plate. Treatments with TGF-β1 were performed as described above. Five wells were handled in parallel and the middle three values were averaged for each condition. Since SNU16 and SNU16α5 cells are mostly (>95 % in population) anchorage-independent and approx. 10 % of SNU16Ad cells are non-adherent, cell adhesion levels were analysed by measuring the absorbance (A) at 564 nm (with a mean value for BSA control wells subtracted) after staining with Crystal Violet. For adhesion assay using transiently transfected cells, cells were first transiently transfected with a haemagglutinin-tagged expression vector encoding either inactive ($Tyr^{416} \rightarrow$ Phe, Y416F) or active (Y527F) c-Src or a Raf-1 mutant not binding to Ras (Raf-1 R89L; a gift from Dr Rudy Juliano, University of North Carolina at Chapel Hill, NC, U.S.A.). Then pretreatment with inhibitors, replating and TGF-β1 treatment were performed as explained above, followed by adhesion analysis.

Determination of Ras-GTP level

To determine Ras-GTP level using GST-RBD (where RBD stands for Ras-binding domain of Raf-1;1–140) and cell lysates, the GST-RBD pull-down assay was performed as described previously [29].

RESULTS

In our efforts to understand how a collaborative signalling of integrin with TGF-β1-mediated signalling pathway modulates cellular functions, we observed that TGF-β1 treatment resulted in an increased adhesion of an anchorage-independent gastric carcinoma cell line, SNU16. To examine which signalling molecules contribute to the TGF-β1-mediated effects, we tested the SNU16 cell line and its subline (SNU16Ad) enriched for adherent cells for adhesion characteristics and intracellular signalling activities.

We first found that SNU16 and SNU16Ad cells had to be replated on fibronectin or other ECM substrates for more than 12 h for a substantial adhesion (results not shown). Therefore, using SNU16 and SNU16Ad cells, we examined the effect of TGF-β1 treatment for 20 h on adhesion on ECM-pre-coated dishes. TGF-β1 treatment at a lower dose (1.0 ng/ml) caused increases in the cell adhesion on fibronectin (with more spreading) of SNU16 and SNU16Ad cells, but the increase was greater in SNU16Ad cells (e.g. 2–4-fold) when compared with SNU16 cells (< 2-fold)

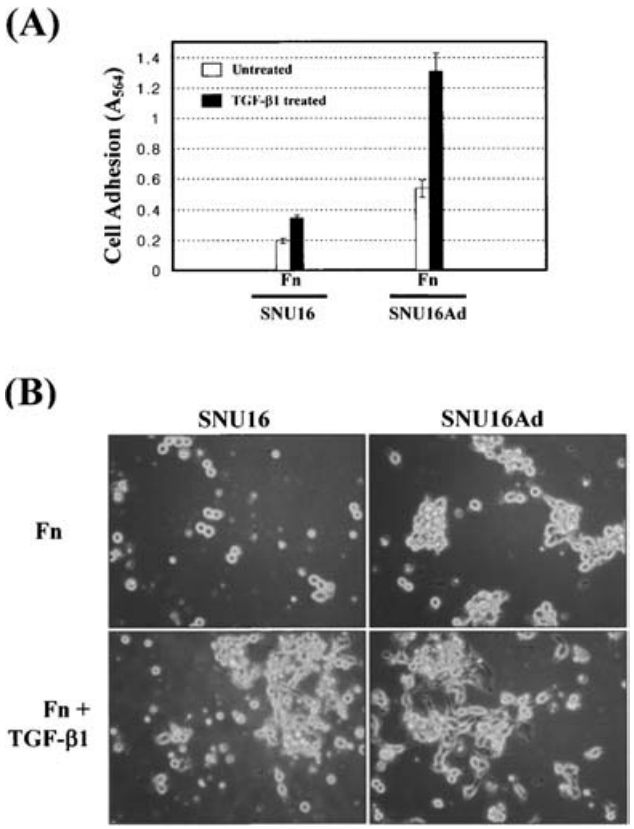

Figure 1 TGF-β1-mediated increase in cell adhesion on fibronectin

(**A**) Predominantly anchorage-independently growing gastric carcinoma cell line (SNU16) and its subline (SNU16Ad) enriched for adherent cells were collected or trypsinized, washed twice with serum-free media containing 1 % BSA, suspended with the same media, counted, rolled at 37 °C for 1 h and then plated at 5×10^4 cells/well on 96-well plates pre-coated with Fn (fibronectin, 15 μg/ml). The cells were treated with 1 ng/ml TGF-β1 for 20 h at the same time of replating. Cell adhesion was measured as described in the Materials and methods section. Results (means $\pm$ S.D.) shown are representative of several independent experiments. (**B**) Phase-contrast images of cells on Fn untreated or treated with TGF-β1. Images shown are representative of several independent observations.

(Figures 1, 3B and 4). TGF-β1-mediated cell adhesion on collagen type I (results not shown) or laminin I (Figure 2C) was very similar to that on fibronectin. To examine whether this increase is mediated by the effects of TGF-β1 on integrin expression, we analysed the levels of integrin subunits (α1, α2, α3, α4, α5, α6, αvβ3, αvβ5, β1 and β4) in SNU16 and SNU16Ad cells by flow-cytometric measurements and Western blotting. The results showed that only the levels of α3 and β1 subunits were higher in SNU16Ad when compared with SNU16 cells, whereas other subunits (α1, α2, α4, α5, α6, αvβ3, αvβ5, β1 and β4) were not changed (Figure 2A, and results not shown). Furthermore, when SNU16 cells were treated with TGF-β1 at 1 ng/ml for 0, 12, 24 or 36 h, the level of integrin α3 subunit alone increased with time among the β1-conjugating integrin subunits we tested (Figure 2B). Therefore we next examined whether the TGF-β1-mediated increase in adhesion is through the integrin α3 subunit. Since integrin α3 binds to both fibronectin and laminin [30,31], blocking of SNU16Ad cell adhesion on both matrix substrates was separately tested by using a functionally blocking anti-α3 antibody (clone P1B5). The basal adhesion was slightly inhibited (with approx. 30 % inhibition) by the functionally blocking anti-α3 antibody, indicating that basal adhesion might involve more integrin subtypes than integrin α3. Meanwhile, the

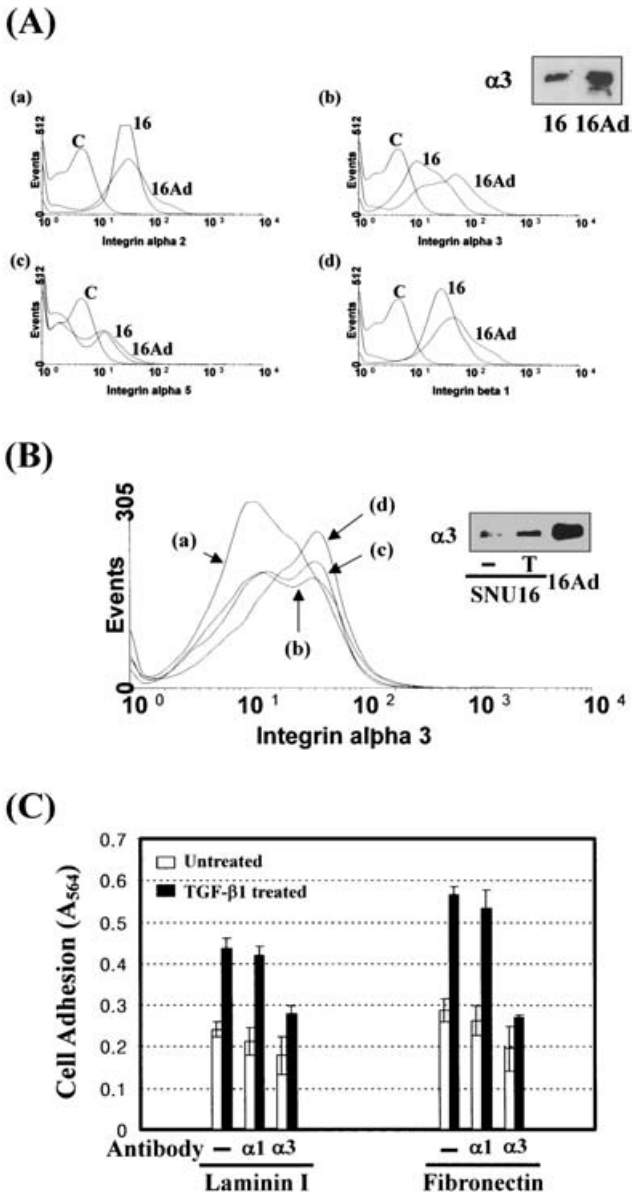

Figure 2 TGF-β1 treatment increases α3 integrin expression in the gastric cells

(**A**) Comparison of integrin expression levels between SNU16 and SNU16Ad cells. SNU16 and SNU16Ad cells were analysed for integrin expression levels by flow cytometry, with respect to integrin α2 (a), integrin α3 (b), integrin α5 (c) and integrin β1 (d). Histograms for negative control (C, without each primary antibody), SNU16 (16) and SNU16Ad (16Ad) cells are shown. Inset shows expression levels of integrin α3 subunit in SNU16 and SNU16Ad cells by a Western-blot analysis. (**B**) Increase in integrin α3 expression level in SNU16 cells by TGF-β1 treatment. Cells were treated with 1 ng/ml TGF-β1 for 0 (a), 12 (b), 24 (c) or 36 (d) h under serum-free condition, before harvests for flow-cytometric measurements. Inset shows expression levels of integrin α3 subunit in SNU16 [untreated or TGF-β1-treated (T) at 1 ng/ml for 20 h] and SNU16Ad cells by a Western-blot analysis. (**C**) TGF-β1-mediated increase in cell adhesion is mediated by integrin α3 subunit increase by TGF-β1 treatment. Functionally blocking anti-α3 (clone P1B5, 27 μg/ml) or control anti-α1 (clone TS2/7, 27 μg/ml) antibodies were preincubated with cells for 1 h at 37 °C in serum-free media containing 1 % BSA before plating of cells on 15 μg/ml fibronectin or laminin. Cell-adhesion assay was performed as described in Figure 1. Results presented are representative of four independent experiments.

TGF-β1-mediated adhesion was abolished by pre-coating cells with the functionally blocking anti-α3 antibody, but not with a control (anti-α1, clone TS2/7) monoclonal antibody (Figure 2C),

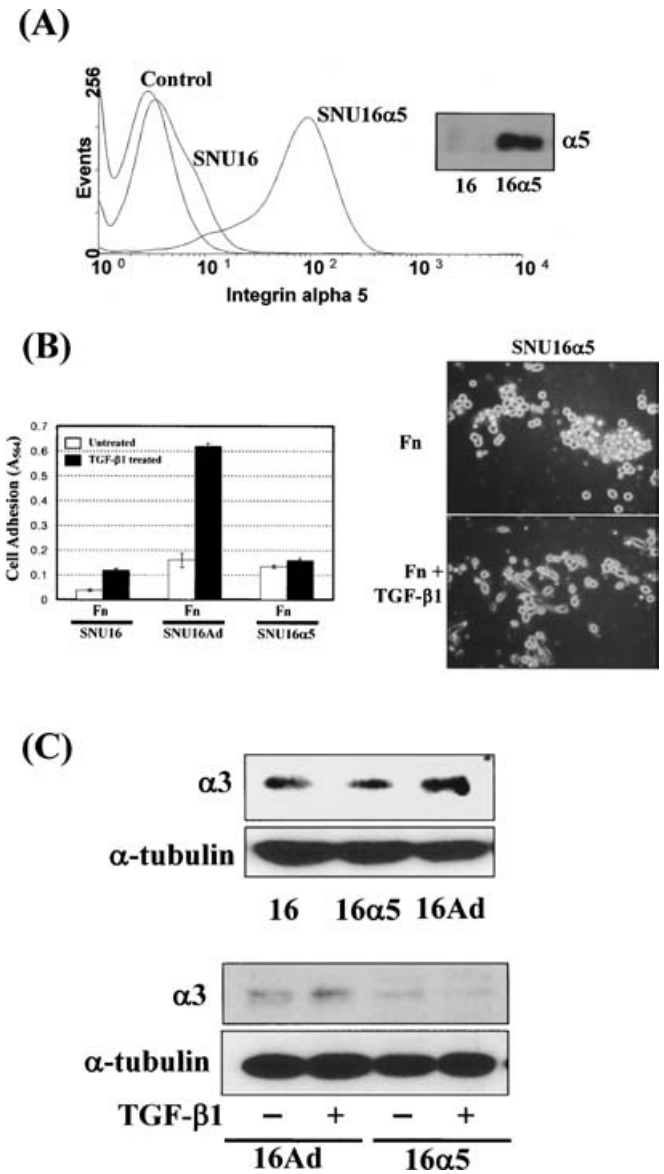

Figure 3 Overexpression of α5 integrin subunit caused neither an increased cell adhesion nor an increased α3 expression level by TGF-β1

(**A**) SNU16α5 cells overexpressing α5 integrin subunit. Flow-cytometric and Western-blot analyses are shown. (**B**) Cell-adhesion analysis (left) of SNU16, SNU16Ad or SNU16α5 cells overexpressing α5 integrin. Replating of cells, treatment with TGF-β1 and measurement of cell adhesion were performed as described in Figure 1. Phase-contrast images (right) of cells on Fn (fibronectin) untreated or treated with TGF-β1. Results shown and images are representative of several independent experiments. (**C**) Expression level of α3 integrin subunit in SNU16α5 cells was not increased by TGF-β1, unlike SNU16Ad cells. Cell lysates were prepared from SNU16 (16), SNU16α5 (16α5) or SNU16Ad (16Ad) cultures with normal serum-containing culture media (upper panel) or from conditions where SNU16Ad (16Ad) or SNU16α5 (16α5) cells were replated on Fn in the absence or presence of TGF-β1 treatment for 20 h. Lysates were used for immunoblots for indicated materials. Results shown are representative of several independent experiments.

indicating that the TGF-β1-mediated increase in adhesion is mediated through integrin α3 subunit, which in turn is increased by TGF-β1 treatment.

Next, to test whether overexpression of other integrin subunits can result in an increased adhesion on fibronectin, cells stably expressing human integrin α5 (SNU16α5) were prepared as a pooled population of α5-positive cells (Figure 3A) and tested for TGF-β1-mediated cell adhesion on fibronectin. However, SNU16α5 cells did not show a TGF-β1-mediated increase in cell adhesion, and their adhesive levels in the absence or presence of TGF-β1 were similar to that of TGF-β1-treated SNU16 cells (Figure 3B). Another α5-positive cell population prepared in parallel showed very similar results (results not shown). Moreover, the α3 expression level in SNU16α5 cells was not higher than that of SNU16 or SNU16Ad cells, and did not further increase on TGF-β1 treatment (Figure 3C), in contrast with SNU16 and SNU16Ad cells (Figures 2B and 3C respectively). These results suggest that the basal and TGF-β1-mediated adhesions of SNU16 and SNU16Ad cells involved increased expression levels of α3 integrin subunit and, presumably, the integrin α3-mediated intracellular signal transduction, being consistent with blocking of the adhesion by a functionally blocking anti-α3 antibody. These findings also suggest that the lack of a TGF-β1-mediated increase in adhesion of SNU16α5 cells might be due to no increase in α3 expression levels, indicating a specific effect by TGF-β1 on avidity of integrin subunits.

It is probable that TGF-β1-mediated regulation of intracellular signalling activities leads to cell adhesion after TGF-β1 treatment. Therefore we next analysed whether pharmacological inhibition of intracellular signalling molecules can alter cell-adhesion properties of SNU16 and SNU16Ad cells. Pharmacological inhibition of c-Src family kinase by PP2, a specific and potent inhibitor, abolished the TGF-β1-mediated increase in cell adhesion (Figure 4). However, a negative control compound of PP2, namely PP3, did not affect the cell adhesion (Figure 4C), suggesting that c-Src family kinase activity is critical for the TGF-β1 effect on adhesion of SNU16 and SNU16Ad cells on fibronectin. Interestingly, inhibition of MEK-1 (MAPK/ERK kinase), and thereby ERK1/ERK2, by U0126 or PD98059 compound did not decrease (but rather slightly increased) the TGF-β1-mediated adhesion, but not the basal adhesion significantly (Figures 4A and 4D), indicating that MEK (and thus ERK1/ERK2) activities had no effect on, or negatively regulated, the TGF-β1-mediated adhesion of SNU16 and SNU16Ad cells. However, inhibition of p38 MAPK or PI3K (and thereby, of PKB/Akt) in both cell lines untreated or treated with TGF-β1 did not alter cell adhesion (Figure 4A), indicating that PI3K and p38 MAPK activities were not involved in the TGF-β1-mediated increase in adhesion of SNU16 or SNU16Ad cells. The cells were treated with 5-aza-2′-deoxycytidine, an inhibitor of DNA methyltransferases that are involved in transcriptional gene silencing, to examine whether (re)induction of genes epigenetically silenced probably during transformation to anchorage independence may cause alteration in cell adhesion. However, 5-aza-2′-deoxycytidine did not cause an increase in cell adhesion of SNU16Ad cells (Figure 4A), suggesting that the TGF-β1-mediated cell adhesion of SNU16 cells and its adherent variant SNU16Ad may involve alteration in signalling activities, rather than epigenetic re-induction of silenced genes under the conditions we tested. Furthermore, the basal and TGF-β1-induced levels of α3 integrin subunit as well were decreased by pretreatment with a specific c-Src family kinase inhibitor (PP2), but not by its negative control compound (PP3) (Figure 4E), indicating that the basal and the TGF-β1-mediated cell adhesions depend on c-Src family kinase activity-dependent levels of integrin α3 subunit.

On the other hand, it is well known that TGF-β1 induces ECM proteins in diverse systems (see the Introduction section). Therefore it is probable that TGF-β1 treatment remodels synthesis and deposition of ECM proteins, such as laminin 5, which is another ligand for integrin α3, leading to an increase in integrin α3-mediated adhesion. To examine this possibility, it was seen if SNU16Ad cells showed the TGF-β1-mediated increase in

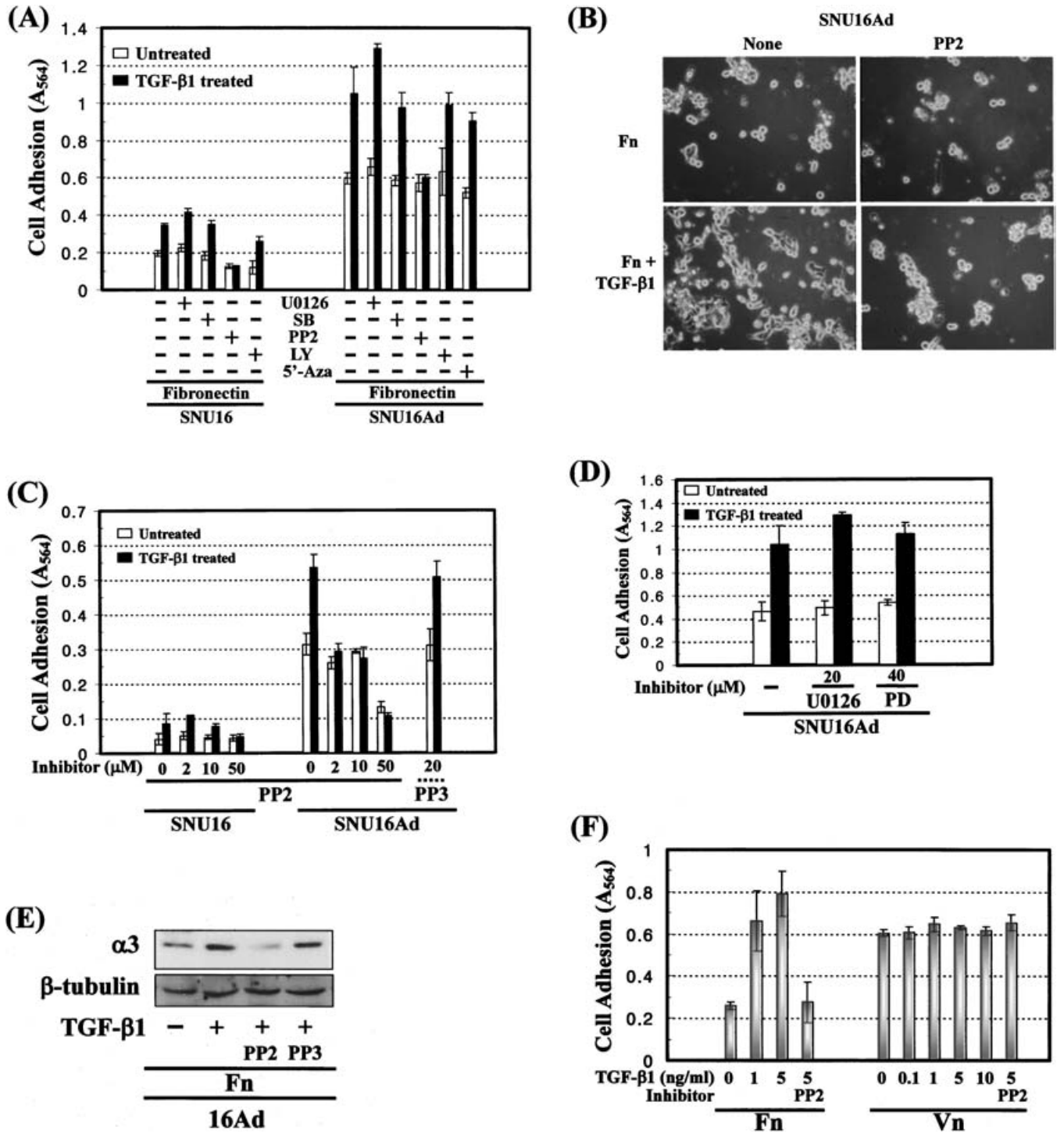

Figure 4 Effects of pharmacological inhibitions on the TGF-β1-mediated increase in cell adhesion

(**A**) SNU16 and SNU16Ad cells were pretreated with U0126 (10 μM), SB203580 (SB, 10 μM), PP2 (10 μM), LY294002 (LY, 20 μM) or 5-aza-2′-deoxycytidine (5-Aza, 100 nM) 30 min before plating. Cell-adhesion assay was performed as described in Figure 1. Results (means $\pm$ S.D.) shown are representative of three independent experiments. (**B**) Phase-contrast images of PP2-pretreated SNU16Ad cells on Fn (fibronectin). Pretreatment with PP2 (10 μM) and cell-adhesion assay were performed as described in (**A**). (**C**) Abolition of the TGF-β1-mediated cell adhesion by c-Src family kinase inhibition. SNU16 or SNU16Ad cells pretreated, or not, with the indicated compounds at various concentrations in the absence or presence of TGF-β1 treatment were analysed for adhesion on Fn as explained earlier. Results (means $\pm$ S.D.) shown are representative of three different experiments. (**D**) Effects of MEKs inhibition on the TGF-β1-mediated cell adhesion. Two different MEK inhibitors, U0126 (20 μM) and PD98059 (PD, 40 μM), were separately pretreated as explained above. Results (means $\pm$ S.D.) shown are representative of two different experiments. (**E**) TGF-β1-mediated increase in α3 expression is abolished by inhibition of c-Src family kinase. SNU16Ad cells were pretreated with none, PP2 or PP3 (a negative control for the c-Src family kinase inhibitor PP2) before replating on Fn in the absence or presence of TGF-β1 treatment. Lysates were used for Western blots for the indicated molecules. Results shown are representative of two different experiments. (**F**) TGF-β1 treatment did not cause an increase in cell adhesion on Vn (vitronectin). Before being replated on either Fn or Vn, cells in certain cases were pretreated with PP2 (10 μM). At the same time of replating, cells were either untreated or treated with TGF-β1 at various concentrations and the incubation lasted for 20 h before adhesion assay as explained in the Materials and methods section. Results (means $\pm$ S.D.) shown are representative of two independent experiments.

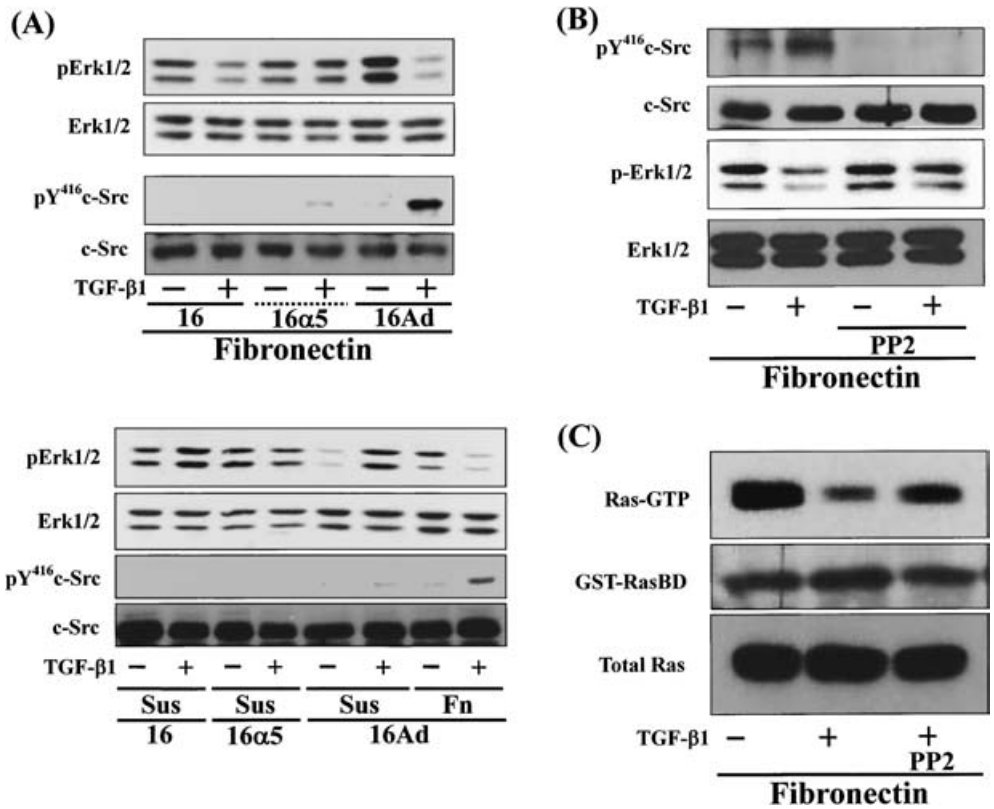

Figure 5 c-Src phosphorylation at Tyr-416 and a concomitant decrease in Ras/ERKs cascade activity on TGF-β1 treatment of SNU16Ad cells

(**A**) Signalling activities of ERK1/ERK2 and c-Src on TGF-β1 treatment to SNU16 (16), SNU16α5 (16α5) and SNU16Ad (16Ad) cells on fibronectin or suspension (sus). TGF-β1 treatment of SNU16, SNU16α5 and SNU16Ad cells was performed by direct addition of TGF-β1 (1 ng/ml) to the media (serum-free media with 1 % BSA) at the same time when cells were kept in suspension or replated on fibronectin for 20 h, before preparation of lysates. The blots shown are representative of several independent experiments. (**B**) A decrease in ERK1/ERK2 activities dependent on c-Src family kinase activity. Replating, pretreatment with the inhibitor PP2 and preparation of lysates were performed as described earlier. Cell lysates were immunoblotted for indicated antibodies. Results shown are representative of three different experiments. (**C**) A decrease in Ras-GTP loading dependent on c-Src family kinase activity. SNU16Ad cell lysates with an equal amount of total protein were used for Ras-GTP determination by the GST-RBD pull-down assay followed by Western blots with anti-Ras antibody or anti-GST (as a control for an equal input of GST-RBD). Lysates were also blotted for an equal amount of total Ras input. Results shown are representative of three different experiments.

adhesion on vitronectin, to which integrin α3 does not bind. If TGF-β1 treatment induced and secreted laminin 5, then cells even on vitronectin had to show an increased adhesion by TGF-β1. As shown in Figure 4(F), however, cells did not show any response to TGF-β1 for adhesion, and c-Src family kinase inhibition did not cause any decrease in cell adhesion. In addition, the immunoblots for laminin 5 showed that TGF-β1 treatment did not cause the production and secretion of laminin 5 (results not shown). The above results, together with the observation shown in Figure 4(E), indicate that the TGF-β1 effects do not involve production and deposition of laminin 5, but involve regulation of integrin α3 expression and thereby, presumably, cellular signal transduction.

Next, intracellular signalling activities of anchorage-independent cell lines or adherent variant cell lines after a TGF-β1 treatment for 20 h were examined biochemically to check how signalling activities differ on TGF-β1 treatment. Although SNU16 and SNU16α5 cells adhered on fibronectin much more weakly than in comparison with SNU16Ad cells, cells were re-plated on fibronectin with a concomitant TGF-β1 treatment and incubated for 20 h at 37 °C before harvests. When parental SNU16 and adherent SNU16Ad cells on fibronectin were treated with TGF-β1, ERK1/ERK2 was inhibited, whereas Tyr-416 phosphorylation of c-Src in SNU16Ad cells on fibronectin was increased (Figures 5A and 5B). Mean while, c-Src phosphorylation at Tyr-416 was predominant when cells on fibronectin were treated with TGF-β1 compared with cells in suspension (Figure 5A, lower panels), indicating that cell-adhesion-mediated signalling might be involved in the c-Src activation by TGF-β1. Interestingly, an inverse relationship between c-Src phosphorylation at Tyr-416 and ERK1/ERK2 activation after TGF-β1 treatment of SNU16Ad cells has been observed (Figures 5A and 5B), i.e. c-Src phosphorylation at Tyr-416 in adherent (i.e. replated) SNU16Ad cells was correlated inversely with ERK1/ERK2 activity after TGF-β1 treatment. Furthermore, pharmacological inhibition of c-Src family kinase also showed the inverse relationship; when c-Src family kinase was inhibited, the TGF-β1-mediated decrease in ERK1/ERK2 activity was abolished (Figure 5B). In addition, consistent with this observation, GTP loading to Ras was decreased by TGF-β1 treatment in a c-Src family kinase activity-dependent manner (Figure 5C). These observations indicate that Ras/ERKs cascade activity in SNU16Ad cells after TGF-β1 treatment was decreased depending on the c-Src family kinase activity, although the relationship among TGF-β1, c-Src family kinases and Ras/ERKs cascade is not clear at present. Together with cell-adhesion results, these results indicate that modulation of the activities of c-Src family kinase and ERK1/ERK2 in an inverse relationship after TGF-β1 treatment may be involved in the TGF-β1-mediated increase in adhesion of SNU16Ad cells.

Next, we examined if the TGF-β1-mediated cell adhesion requires the activity of c-Src and c-Src activity-dependent decrease in ERK1/ERK2 activity, by using transient-transfection experiments. LIPOFECTAMINE™ 2000 was used to transfect SNU16Ad cells, and the transfection efficiency was 30–40% when the cells were checked for expression of green fluorescent protein (results not shown). As shown in Figure 6(A), TGF-β1-mediated cell adhesion was decreased (but incompletely, probably due to an incomplete transfection efficiency) by expression of an inactive c-Src (Y416F), whereas cell adhesion was increased by the introduction of an active form of c-Src (Y527F) even in the absence of TGF-β1 treatment, indicating that the TGF-β1 effects require c-Src activation (Figure 6A). Furthermore, when cells express a mutant of Raf-1 not binding to Ras, the TGF-β1-mediated adhesion was not decreased, or was at best slightly increased, being consistent with adhesion under pharmacological inhibition of MEKs (i.e. ERK1/ERK2) as shown in Figures 4(A) and 4(D) (see also Figure 6B). In addition, the TGF-β1 effects were significantly abolished by PP2, but not significantly by PP3 control compound, indicating again that the TGF-β1 effects involve a decrease in Ras/ERK1/ERK2 cascade signalling activity, depending on the c-Src family kinase activity (Figure 6B). Moreover, the inverse relationship between c-Src and ERK1/ERK2 activities was biochemically examined. The basal and the TGF-β1-mediated levels of ERK1/ERK2 activities in cells expressing active c-Src became much lower in the absence or presence of TGF-β1 treatment, compared with those in control cells transfected with none (Figure 6C).

Taken together, the present study showed a good correlation between the TGF-β1-mediated adhesion of SNU16Ad cells and an increase in integrin α3 subunit level, c-Src family kinase activity and c-Src family kinase activity-dependent decrease in Ras/ERKs cascade activity.

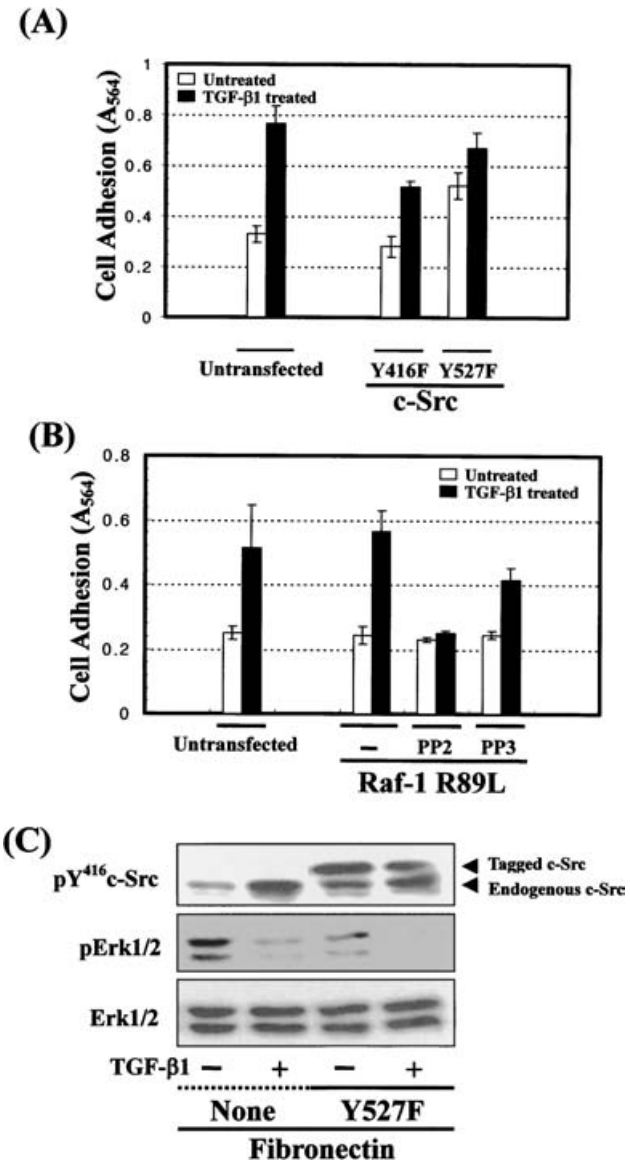

Figure 6 An inverse relationship between c-Src and ERK1/ERK2 activities is required for the TGF-β1-mediated SNU16Ad cell adhesion

(**A**) Requirement of c-Src activity for the increased cell adhesion on TGF-β1 treatment. Cells were transiently transfected with plasmids encoding either inactive (Y416F) or active form (Y527F) of c-Src. Cells were replated on fibronectin 20 h after transfection. Cells were treated with 1 ng/ml TGF-β1 for 20 h and cell-adhesion analysis was performed as described in Figure 1. Results (means $\pm$ S.D.) shown are representative of two different experiments. (**B**) Requirement of c-Src family kinase activity-dependent decrease in Ras/Raf-1/ERKs cascade activity for the TGF-β1 effects. Cells were transiently transfected with an expression vector for a mutant of Raf-1 not binding to Ras (Raf-1 R89L). Cells were treated with TGF-β1 and cell-adhesion analysis was performed as described in (**A**). The c-Src family kinase inhibitor PP2 (20 μM) or its negative control (PP3, 20 μM) was pretreated before replating and concomitant TGF-β1 treatment as described earlier. Results (means $\pm$ S.D.) shown are representative of two independent experiments. (**C**) Inverse relationship between c-Src and ERK1/ERK2 activities. Cells untransfected or transfected with active c-Src (Y527F) were performed as in (**A**) and harvested, and the lysates were used for the immunoblots for the indicated molecules. Results shown are representative of two independent experiments.

DISCUSSION

In our efforts to study how a signalling cross-talk between integrin and TGF-β1 pathways influences cellular functions, we observed that TGF-β1 treatment induced an increased adhesion of the anchorage-independent SNU16 gastric carcinoma cells. We studied the SNU16 cell line and its subline (SNU16Ad) enriched to be adherent to determine signalling molecules that underlie the TGF-β1 effects. We observed that treatment with TGF-β1 increased the expression level of integrin α3 subunit, resulting in an unusual inverse relationship between c-Src family kinase and ERK1/ERK2 on TGF-β1 treatment; c-Src family kinase was activated but, concomitantly, Ras/ERKs cascade activity was decreased, depending on the c-Src family kinase activity. Furthermore, it was shown that an increased level of integrin α3 expression and the inverse relationship between c-Src family kinase activity and Ras/ERKs cascade activity were well correlated with the TGF-β1-mediated increase in cell adhesion.

Since TGF-β1 treatment of parental SNU16 and its adherent variant SNU16Ad resulted in increased adhesion, we analysed SNU16 and SNU16Ad cells to study signalling molecules involved in cell adhesion during integrin co-signalling with TGF-β1 pathways. We found that TGF-β1 treatment of SNU16 cells resulted in an increase in the level of integrin α3 subunit, but not of other major β1-associated integrin subunits. Similarly, SNU16Ad cells express more integrin α3 subunit, but not other subunits, when compared with SNU16 cells. TGF-β1-mediated increase in integrin α3 subunit level correlates well with the TGF-β1-mediated increase in adhesion on laminin 1, collagen or fibronectin. In addition, the TGF-β1-mediated increase in adhesion and the basal adhesion were slightly inhibited by precoating cells with a functionally blocking anti-α3 antibody, as well as by a specific c-Src family kinase inhibitor, indicating that a specific integrin subtype-mediated intracellular signalling pathway underlies the increased cell adhesion induced by TGF-β1

treatment. Being consistent with c-Src activity-dependent cell adhesion, not only the basal level but also TGF-β1-mediated increases in the level of integrin α3 subunit in SNU16Ad cells were abolished by c-Src inhibition. These results together with inhibition of the basal and the TGF-β1-mediated cell adhesion by anti-α3 antibody indicate a good relationship among TGF-β1-mediated c-Src activity, integrin α3 expression and cell adhesion. We also examined whether an increased expression of another integrin subtype is sufficient to allow the TGF-β1-mediated change in cell adhesion; i.e. the specificity of the TGF-β1 effects was tested. However, overexpression of integrin α5 subunit was not enough to cause a significantly increased adhesion on normal cell culture dishes (results not shown). Moreover, α5-expressing cells (SNU16α5) did not respond to TGF-β1 treatment for an additional cell adhesion and for an increase in integrin α3 level (Figure 3). However, SNU16Ad cells showed a c-Src activity-dependent expression level of integrin α3 and cell adhesion (Figures 4A–4C and 4E). These results indicate that overexpression of any integrin is not sufficient for increased adhesion of SNU16Ad cells by TGF-β1, again supporting the idea that the TGF-β1 effects might involve regulation of integrin α3 expression and, thereby, specific integrin-mediated signal transduction. An alternative explanation, in which TGF-β1-mediated synthesis and deposition of ECM proteins such as laminin 5 may lead to an increased adhesion, was excluded by the observations that SNU16Ad cells on vitronectin (not adhesive to integrin α3) were not responsive to TGF-β1 for additional cell adhesion (Figure 4F), and that laminin 5 was not detected biochemically when SNU16Ad cells replated on fibronectin were treated with TGF-β1 (results not shown).

The effects of TGF-β1 treatment on integrin expression levels may be specific to the cell types. Previously, TGF-β1 treatment of a human osteosarcoma cell line resulted in a decrease in integrin α3 mRNA and protein levels with concomitant increase in integrin α2, α5 and β1 levels [32]. In addition, a recent report [33] also showed that integrins α3 and α6 were decreased, whereas integrins α2 and α5 were increased on TGF-β1 treatment of keratinocytes. On the other hand, another study [34] reported that TGF-β1 treatment of non-invasive human hepatocellular carcinoma showed a significant increase in integrin α3 level with concomitant increases in integrin α2, β1 and β4 subunit levels. However, in the present study, only integrin α3 and β1 subunits were increased by TGF-β1 treatment, but other integrin subunits tested (α1, α2, α4, α5, α6, αvβ3, αvβ5 and β4) were not changed. These results, of increase in integrin α3 expression level and blocking of the TGF-β1-mediated adhesion by anti-α3 antibody, strongly suggest that the effects of TGF-β1 on integrin expression and thereby cellular behaviour appear to be quite specific.

Previous studies [35,36] have reported that TGF-β1 treatment of NRK (normal rat kidney) fibroblasts resulted in the induction of integrin α5 subunit and cyclin D1, leading to anchorage-independent growth. Furthermore, a restored surface expression of integrin α5 subunit preceded the induction of anchorage-independent growth [35]. In contrast, in the present study, TGF-β1 treatments of SNU16 or SNU16Ad cells did not alter the levels of integrin α5 subunit or cyclin D1 (results not shown) and the cells became adherent on TGF-β1 treatment, unlike the NRK cells, which became anchorage-independent. This difference may be due to different cell types and/or different behaviour of parental cells. In contrast with adherent NRK fibroblasts, the present study has used an anchorage-independent gastric carcinoma cell line and its adherent variant. Meanwhile, overexpression of integrin α5 subunit in anchorage-independent SNU16 cells did not result in any significant increase in the adherent population during normal culture. Most cells (SNU16α5) still grew in an anchorage-independent manner (results not shown), although they showed an increased basal adhesion on being replated on fibronectin, but not the TGF-β1-mediated increase in adhesion. SNU16α5 cells in the absence of TGF-β1 showed a slightly less, or at best a similar, adhesion level when compared with SNU16Ad cells (Figure 3B), but they did not show the TGF-β1-mediated increases in adhesion and in integrin α3 level, unlike SNU16Ad cells (Figures 3B, 3C and 4E). Therefore it is probable that responsiveness to TGF-β1 for an increase in α3 integrin was lacking in SNU16α5 cells, and thus, no TGF-β1-mediated increase in adhesion was observed.

Previously, it was shown that TGF-β1 modulates c-Src non-receptor tyrosine kinase activity negatively or positively depending on cell types and/or probably the nature of its treatment. The present study showed that c-Src activation by TGF-β1 appears to depend on cell-adhesion status; TGF-β1 treatment did not result in significant phosphorylation of Tyr-416 of c-Src in suspended cells, whereas it caused a significant increase in adherent cells (Figure 5A). Similar to the present study, TGF-β1 treatment to HaCaT human keratinocytes, MDCK (Madin–Darby canine kidney) epithelial cells and Mahalavu hepatoma cells resulted in increases in c-Src activity [37,38]. In those studies, however, TGF-β1 treatments were performed for a short time such as 15 min, unlike the present study where the treatment was performed for 20 h even without any significant damage to or death of cells (results not shown). On the other hand, in v-Src-transformed rat fibroblasts, HepG2 hepatoma cells and human prostate carcinoma cell line PC3, TGF-β1 treatment caused a decrease in c-Src activity and protein abundance [38,39]. Therefore the effects of TGF-β1 on tyrosine kinase c-Src may depend on the cell types and/or signal properties on TGF-β1 treatments.

c-Src has been implicated in cell adhesion in many model systems [40–42]. However, it is not clear whether c-Src activity is required for cell adhesion. Src-null fibroblasts were defective in cell adhesion, but the c-Src expression in Src-null fibroblasts promoted cell adhesion even without the c-Src kinase activity [43]. However, other studies showed that cell adhesion involves c-Src activity-dependent mechanisms. Integrin-mediated migration and adhesion of fibroblasts required c-Src activity but not the scaffolding function by its SH2 and/or SH3 domain-mediated intermolecular interaction [42]. Furthermore, in fibroblasts, c-Src activity was needed for focal adhesion formation during cell-adhesion processes through Rap1 activation [41]. Similar to the latter cases, the present study showed that endogenous c-Src family kinase activity was well correlated with the increased adhesion of SNU16 and SNU16Ad cells after TGF-β1 treatment (Figures 4 and 5), and that c-Src inhibition by pretreatment with a pharmacological inhibitor or transfection of mutants of c-Src blocked the basal and the TGF-β1-mediated cell adhesion (Figure 6A).

Previously, most studies showed that TGF-β1 treatment resulted in activation of ERK1/ERK2, and the mediator between TGF-β1 receptors and ERK1/ERK2 cascade appears to involve Ras protein [44–46]. On the other hand, similar to this study, inhibitory effects of TGF-β1 on ERK1/ERK2 activity have also been reported. In a study using smooth-muscle cells, ERK2 activation by basic fibroblast growth factor was inhibited by TGF-β1 pretreatment; interestingly, a shorter (40 min) pretreatment with TGF-β1 resulted in a decrease in threonine phosphorylation of ERK2, indicating an action of a serine/threonine phosphatase, whereas a longer pretreatment (4 h) decreased both threonine and tyrosine phosphorylation levels, indicating a blocking of the ERK cascade or directly ERK itself [47]. Therefore the effects of TGF-β1 treatment on mitogenic activity indicated by ERK1/ERK2 activity may be different, depending on the treatment period

and/or cell types. In an earlier study using mesangial cells, v-Src activation was correlated with suppression of ERK1/ERK2 by unknown mechanisms [48]. In addition, in this study, c-Src activity was well correlated with increased cell adhesion and with decreased ERK1/ERK2 activity on TGF-β1 treatment as well. By pharmacological inhibition and transient-transfection studies, TGF-β1-mediated adhesion was abolished or rather slightly increased by inhibition of c-Src family kinase or Ras/ERKs cascade activity respectively. In addition, both Ras-GTP loading and ERK1/ERK2 activity were partially restored to the level of untreated cells in a c-Src family kinase activity-dependent manner. Furthermore, expression of active c-Src into SNU16Ad cells resulted in decreases in ERK1/ERK2 activities in the absence or presence of TGF-β1 treatment (Figure 6C), indicating that activity status of c-Src is inversely related to ERK1/ERK2 activity in this system. Thus the present study supports the idea that c-Src family kinase activity is required for TGF-β1-mediated increases in integrin α3 level and in cell adhesion of SNU16 and SNU16Ad cells, and suggests that there is an inverse relationship between c-Src family kinase and Ras/ERKs cascade during the TGF-β1-mediated cell adhesion.

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