Overexpression of the C-terminal PG-M/versican domain impairs growth of tumor cells by intervening in the interaction between epidermal growth factor receptor and β_1-integrin

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Summary

Versican is highly expressed in many types of tumors. In a previous study, we found that a G3 mutant [G3ΔEGF; a versican G3 domain lacking two epidermal growth factor (EGF)-like motifs] exerted a dominant-negative effect on versican secretion and binding. Here, we report that astrocytoma U87 cells expressing the versican G3 mutant lost the hallmark of cell transformation and tumorigenesis in vitro and in vivo. U87 cells expressing G3ΔEGF had enhanced cell adhesion and spreading, but lost the tumor characteristic of anchorage-independent growth. When U87 cells were deprived of serum, FAK was quickly dephosphorylated, integrin/EGF-receptor (EGFR) complexes dissociated and the cells retained an appropriate level of EGFR phosphorylation. These cells quickly detached, migrated, rounded, reorganized and survived. However, after serum withdrawal from G3ΔEGF-transfected U87 cells, sustained FAK phosphorylation and integrin-EGFR association were observed, but a greatly reduced EGFR phosphorylation. These cells remained spread and continued to grow before undergoing massive apoptosis. The addition of EGF promoted U87 cell rounding but had little effect on G3ΔEGF-transfected cells owing to reduced EGFR phosphorylation. Our study sheds light on the question of how the matrix molecule versican modulates tumorigenesis by affecting integrin and EGFR signals.

Supplemental data available online

Key words: EGFR, G3 domain, Phosphorylation, Interaction, Cell cycle, Immunoprecipitation

Introduction

Versican is a member of the large aggregating chondroitin sulfate proteoglycan family. It was originally isolated from human fibroblasts and developing limb buds in the chicken (Shinomura et al., 1993; Zimmermann and Ruoslahti, 1989), and was later detected in brain tumors (Paulus et al., 1996). Structurally, versican is made up of an N-terminal G1 domain, a glycosaminoglycan (GAG) attachment region and a C-terminus containing a selectin-like (or G3) domain. The G3 domain contains two epidermal growth factor (EGF)-like repeats, a lectin-like motif (also known as carbohydrate recognition domain or CRD) and a complement binding protein (CBP)-like motif (Shinomura et al., 1993; Zimmermann and Ruoslahti, 1989). Alternative splicing generates at least four versican isoforms (Ito et al., 1995; Lemire et al., 1999), and some of these are highly expressed in tumor tissues (Cattaruzza et al., 2002; Paulus et al., 1996). Versican is known to associate with several molecules in the extracellular matrix (ECM) including hyaluronan, tenascin, fibulin-1, fibrillin, fibronectin, CD44 and L-selectin (Aspberg et al., 1995; Isogai et al., 2002; Kawashima et al., 2000; LeBaron et al., 1992; Olin et al., 2001).

Integrins are the major cell surface receptors expressed by all cell types. They are composed of α and β transmembrane subunits (Couzin, 2001). Each αβ combination has its own binding specificity and signaling properties. Incorporated with various α subunits, β_1-integrin binds to diverse ECM molecules (Yokosaki et al., 1998). The fundamental cellular function of integrins is adhesion and they mediate extensive and important cellular functions by interacting with the ECM (Condic and Letourneau, 1997). These interactions are prominent activators of signal transduction.

It has been known that integrins are able to form clusters with the EGF receptor (EGFR), and that this physical interaction frequently affects the intensity of signals downstream from the EGFR, such as extracellular-signal-regulated kinase (ERK) (Yamada and Even-Ram, 2002). Focal-adhesion kinase (FAK) is associated with integrin within focal adhesions, and integrin activation by ECM ligands is associated with increased tyrosine phosphorylation and kinase activity of FAK (Kornberg et al., 1992). The activation of FAK plays an important role in integrin-mediated cell adhesion and spreading. By contrast, FAK phosphorylation is also regulated by the EGFR (Lu et al., 2001).

We have previously demonstrated that versican stimulates cell proliferation and migration, while inhibiting cell adhesion and differentiation (Ang et al., 1999; Yang et al., 1999; Zhang et al., 1998a; Zhang et al., 1998b). However, the signaling pathway for this role is not known. Recently, we observed that a mutant versican exerts a dominant-negative effect on cell proliferation by inhibiting the secretion of endogenous versican and can interact with astrocytoma cells (Wu et al., 2001). The G3 mutant interacts with β_1-integrin, induced enhanced β_1-integrin expression and FAK phosphorylation with increased cell adhesion (Wu et al., 2002). The purpose of this study was to investigate the possible activity of versican in the common EGFR- and integrin-mediated pathway.

Materials and Methods

Materials

Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), Hank's balanced salt solution (HBSS), trypsin/EDTA and Geneticin (G418) were purchased from Invitrogen. The ECL western blot detection kit was from Amersham. Antibodies against EGFR and phosphorylated EGFR were from Santa Cruz Biotechnology. Horseradish-peroxidase-conjugated goat anti-mouse IgG secondary antibody was from Sigma (St Louis, MO). Tissue-culture plates were from Nunc. All other chemicals were from Sigma. The U87 astrocytoma cell line, COS-7 cells and Jurkat cells were obtained from the American Type Culture Collection (Rockville, MD).

Construction and expression of recombinant construct

A recombinant construct (G3ΔEGF) containing the link protein leading peptide and the versican G3 domain lacking two EGF-like motifs was used in this study. This construct was generated previously (Chen et al., 2003; Kiani et al., 2003; Yang et al., 2000). The link protein leading peptide contains an epitope recognized by the monoclonal antibody 4B6 (Binette et al., 1994). The G3ΔEGF construct was stably expressed in the astrocytoma cell line U87. Products secreted to conditioned medium were harvested and analysed by western blotting to confirm the presence of the G3ΔEGF product. In our previous studies, we have demonstrated that stable expression of G3ΔEGF decreases cell proliferation when cultured under low-serum conditions (Wu et al., 2001). Also, the selection marker G418 must be included in the culture medium at all times to ensure that the G3ΔEGF construct is well expressed. Vector-transfected U87 cells were used as an appropriate control.

Cell cultures

In serum-free cultures, confluent U87 cells stably transfected with a vector or G3ΔEGF were starved overnight, then harvested and suspended in serum-free DMEM. These cells were seeded on tissue-culture plates in serum-free medium and incubated at 37°C for different times to examine changes in cell morphology, growth and survival. In serum-withdrawal cultures, equal numbers of cells were first cultured in DMEM containing 10% FBS for 24 hours. The medium was then changed to serum-free DMEM and cells were incubated at 37°C for different times to examine changes in cell morphology, growth, cell cycle, apoptosis and expression levels of FAK, EGFR and β_1 integrin.

Colony formation and tumorigenesis

Colony formation was assessed using a method previously described (Busse et al., 2000). Briefly, 10^3 cells were mixed in 0.4% agarose in DMEM supplemented with 10% FBS and plated onto 0.6%-agarose-coated six-well tissue-culture plates. 3 weeks after cell inoculation, colonies were examined and photographed. Those having a diameter of ≥50 μm were counted. In the tumor formation assay, 6-week-old nude mice (strain Balb/c, from Charles River) were injected subcutaneously in the left or right flank with G3ΔEGF- and vector-transfected U87 cell lines (10^7 cells in 200 μl PBS for each mouse). Tumor size was measured 7 weeks after injection. Each cell line was tested in three individuals and the experiments were repeated three times. Tumor volume (V) was estimated using a caliper by measuring the length (L) and width (W): $V=(L\times W^2)/2$.

Cell proliferation and apoptosis

2×10^5 cells per well of vector- and G3ΔEGF-transfected U87 cells were seeded to six-well plastic tissue-culture plates (–agarose) or to six-well plates coated with 0.6% agarose (+agarose) in triplet wells in DMEM containing 10% FBS, and incubated for different times. The cell numbers were counted. For cell cycle analysis, fully confluent cells were synchronized by starvation and 2×10^5 cells were seeded on six-well tissue-culture plates. After treatment, the cells were collected following trypsinization, pelleted by centrifugation and resuspended in 1 ml hypotonic propidium iodide solution (50 μg ml^{-1}) dissolved in 0.1% sodium citrate plus 0.1% Triton X-100. The cells were analysed using fluorescence-activated cell sorting in a FACScan. In the apoptosis assay, after treatment and incubation for different times, the cells were subjected to Annexin-V/FITC staining (15 minutes in the darkness) using an Annexin V apoptosis detection kit (Santa Cruz Biotechnology) and the apoptotic cells were quantified using a FACScan. Trypan blue staining was also used to determine cell viability.

Immunofluorescence

Cells were grown on glass coverslips at 10% density in DMEM supplemented with 10% FBS, incubated for 24 hours and then starved in serum-free DMEM for 24 hours. The cultures were fixed with 4% paraformaldehyde, permeabilized in PBS containing 0.2% Triton X-100 and blocked with 5% bovine serum albumen (BSA). Cells were incubated with TRITC-labeled phalloidin to detect actin fibers and then with anti-vinculin antibody at room temperature for 1 hour, washed and incubated with FITC-conjugated goat anti-mouse IgG secondary antibody. After a final wash and mounting, cells were examined using a laser confocal microscope.

Immunoprecipitation

Cell lysate was prepared in lysis buffer for protein interaction (20 mM Tris-Cl, pH 7.4, 150 mM NaCl, 2 mM PMSF, 10 μg ml^{-1} leupeptin, 2 mM EDTA, 5 μg ml^{-1} BSA and 1.5% Triton X-100) and clarified by centrifugation at 12,000 ***g*** for 40 minutes at 4°C. The supernatant was incubated at 4°C overnight with protein G that had been saturated with anti-EGFR monoclonal antibody, followed by blocking with 5% BSA. After washing, the beads were boiled in 1× protein loading dye for 5 minutes and analysed by western blotting using an anti-β_1-integrin antibody (Chemicon International, clone JB1a).

EGFR and FAK phosphorylation

Equal numbers of G3ΔEGF- and vector-transfected U87 cells were seeded on six-well tissue-culture plates at 20-50% densities in DMEM supplemented with 10% FBS, and incubated for 24 hours. The culture medium was changed to serum-free DMEM and the cells incubated for different times. Cell lysate was prepared in a lysis buffer for protein phosphorylation detection containing 50 mM HEPES, pH 7.5, 0.5% Triton X-100, 100 mM NaF, 10 mM sodium phosphate, 4 mM EDTA, 2 mM sodium vanadate, 2 mM sodium molybdate, 2 μg ml^{-1} aprotinin, 2 μg ml^{-1} leupetin and 2 μg ml^{-1} PMSF, and subjected to

analyses of EGFR and FAK tyrosine phosphorylation. For EGFR phosphorylation, cell lysate was also prepared from the cells treated with EGF at 100 ng ml^{-1} for 0, 5, 20 and 40 minutes after starvation for 24 hours in the serum-free DMEM. The cell lysate was clarified by centrifugation. The supernatant was incubated with protein G that had been pre-saturated with anti-EGFR or anti-FAK monoclonal antibody at 4°C overnight. After washing, the beads were boiled in 1× protein loading dye for 5 minutes, followed by western blotting probed with anti-phosphotyrosine antibody (clone PY-20; Transduction Laboratories). The protein levels of EGFR and FAK were examined by probing the same filters used for protein phosphorylation analysis (after stripping) with anti-EGFR or anti-FAK antibody, or by parallel western blot analysis using the same cell lysate that was being used for protein phosphorylation detection.

Effects of AG1478 on EGFR phosphorylation, cell proliferation and apoptosis

To examine the effect of AG1478 on EGFR phosphorylation, 2×10^5 cells were seeded on six-well plates and incubated in 10% FBS in DMEM overnight, followed by starvation in serum-free DMEM for 24 hours. The cultures were treated with or without 20 μM AG1478 for 30 minutes, followed by incubation with 100 ng ml^{-1} EGF for 30 minutes. The cells were lysed with a lysis buffer for protein phosphorylation detection as described above and subjected to western blotting probed with anti-phospho-EGFR antibody. In a proliferation assay, cells were grown to confluence with 10% FBS in DMEM and then rendered quiescent with serum-free DMEM overnight. The cells were harvested and inoculated at a cell density of 1×10^5 cells per well on 12-well tissue-culture plates in 1% FBS in DMEM containing 20 μM AG1478 dissolved in dimethyl sulfoxide or containing dimethyl sulfoxide alone, and incubated for 3 days to test the effect of AG1478 on cell proliferation. In the cell apoptosis assay, cells were treated with AG1478 for 5 days followed by staining with annexin V-FITC and quantified using a FACScan.

Statistical analysis

The results (mean values±SD) of all of the experiments were subjected to statistical analysis by *t*-test. The level of significance was set at $P<0.05$.

Results

Loss of cell transformation and tumorigenesis caused by versican C-terminal domain

We have previously observed that a C-terminal fragment containing the CRD and CBP motifs (G3ΔEGF construct; Fig. 1A) inhibits the secretion of endogenous versican and reduces cell growth (Wu et al., 2001). Here, when cultures were overgrown, the vector-transfected cells formed nodules on the plates (Fig. 1B). However, the G3ΔEGF-transfected U87 cells did not form nodules; they could only be grown as a monolayer. Analysis of the growth curve indicated that the G3ΔEGF-transfected cells had reached a plateau and could no longer proliferate, whereas vector-transfected cells continued to proliferate after the cultures became confluent (Fig. 1C). To examine anchorage dependence in growth, the cells were cultured on agarose-coated plates, thus being deprived of potential attachment to substratum. The G3ΔEGF-transfected cells did not grow, whereas the vector-transfected cells proliferated (Fig. 1D) and formed large aggregates in

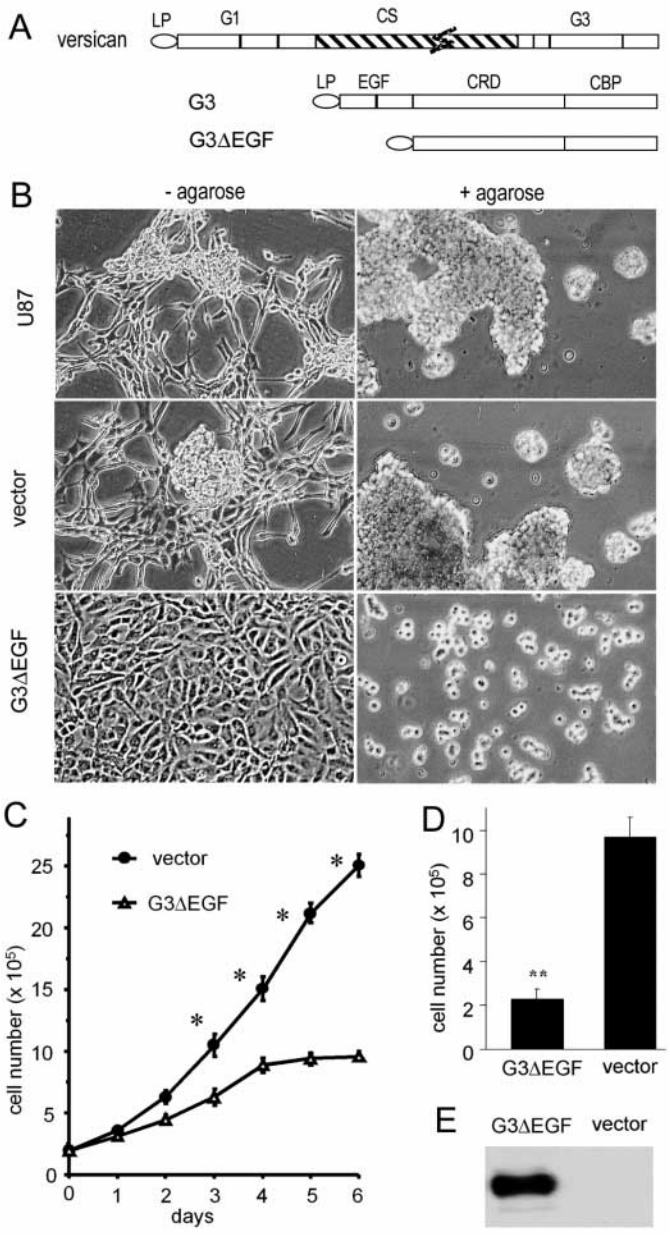

Fig. 1. Expression of G3ΔEGF resulted in cell contact inhibition and reduced cell proliferation. (A) Structures of versican, the versican G3 domain and the G3 domain lacking two EGF-like motifs (G3ΔEGF). (B) Vector- and G3ΔEGF-transfected, and untransfected U87 cells (2×10^5 cells per well) were cultured on six-well tissue-culture plates to obtain monolayer cultures (–agarose) or on six-well plates coated with 0.6% agarose gel to obtain suspension cultures (+agarose). In the monolayer cultures, the vector-transfected and untransfected cells formed nodules, whereas the G3ΔEGF-transfected cells could only form a monolayer owing to growth contact inhibition. On the agarose-coated plates, the vector-transfected and untransfected cells formed large complexes, whereas the G3ΔEGF-transfected cells formed small conglomerates. (C) Cell growth curves showed that, under monolayer culturing conditions, transfection with G3ΔEGF resulted in a plateau after the cultures reached confluence. (D) On the agarose-coated plates, the vector-transfected cells exhibited higher rate of proliferation than the G3ΔEGF-transfected cells. (E) Expression of G3ΔEGF was confirmed by western blot probed with the monoclonal antibody 4B6. All cell numbers are expressed as the means±SD of three experiments, each performed in triplicate wells,. *$P<0.01$; **$P<0.001$.

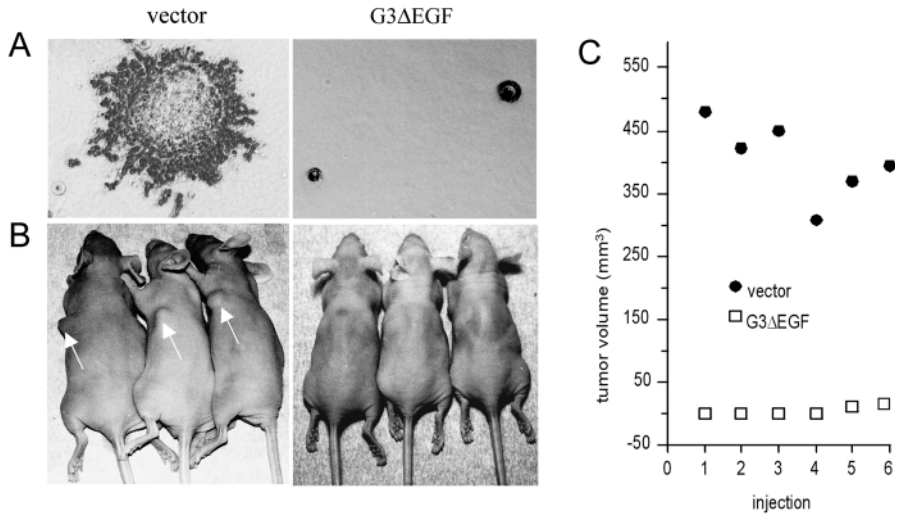

Fig. 2. Colony formation and tumorigenesis assays. (A) Cells were grown in soft agarose gel. Vector-transfected U87 cells formed colonies but G3ΔEGF-transfected U87 cells did not. Each treatment was done in triplicate wells and the experiment was repeated twice. (B) Both vector- and G3ΔEGF-transfected cells were injected into the nude mice at 10^7 cells per mouse. Six weeks after injection, tumors were formed by vector-transfected cells (arrows) but not by G3ΔEGF-transfected cells. The photos are results from one experiment. The tumor sizes were measured, and the results from two experiments are given in (C).

suspension culture (Fig. 1B). Other stably G3ΔEGF-transfected U87 cell lines also produced similar results (data not shown). Little clonal variation was observed, consistent with our previous reported role of G3ΔEGF in cell proliferation (Wu et al., 2001). Expression of the G3ΔEGF construct was confirmed using western blotting probed with the monoclonal antibody 4B6, which recognizes an epitope in the leading peptide of the construct (Fig. 1E).

We examined the two types of cells for differences in colony formation in soft agarose gel and observed that the vector-transfected cells formed large colonies (82.7±6.9 colonies per plate, n=3), whereas no major colonies were detected in G3ΔEGF-transfected cells (Fig. 2A). We further examined the effect of G3ΔEGF-expressing cells on tumorigenicity in vivo. U87 cells, stably transfected with either G3ΔEGF or the control vector, were introduced into nude mice. Vector-transfected cells formed tumors but G3ΔEGF-transfected cells did not (Fig. 2B). This experiment was repeated three times with different stably transfected cell lines and gave rise to similar results (Fig. 2C). These results indicated that G3ΔEGF expression changed U87 cell phenotype and significantly affected their tumorigenicity.

G3ΔEGF enhances serum-depletion-induced apoptosis and reduces EGFR phosphorylation

We next examined the effect of G3ΔEGF on cell morphology at high and low levels of serum using a serum withdrawal assay. G3ΔEGF- and vector-transfected U87 cells were seeded on tissue-culture plates at 20% density in DMEM containing 10% FBS. 1 hour after inoculation, G3ΔEGF-transfected cells exhibited spreading on the plates, whereas vector-transfected cells had just begun to attach (Fig. 3A). After 24 hours of incubation, serum was withdrawn from the G3ΔEGF- and vector-transfected cell cultures by changing the regular medium to serum-free DMEM. 3 hours after the medium was changed, vector-transfected cells showed reduced spreading but the G3ΔEGF-transfected cells did not (data not shown). At 24 hours, the bodies of vector-transfected cells retracted, but some of the processes elongated and connected to other cells. The G3ΔEGF-transfected cells, however, remained spread on the plates (Fig. 3B). The cell numbers in the vector-transfected cultures declined slowly over 4 weeks, whereas the numbers in G3ΔEGF-transfected cells increased over the first 5 days, followed by a dramatic decline owing to cell death (Fig. 3C). The proportion of dead cells increased every day in the G3ΔEGF-transfected cultures (Fig. 3D). Flow cytometry analyses indicated that G3ΔEGF-transfected cells had a higher rate of apoptosis than the vector-transfected cells (data not shown). Cell cycle analyses indicated little difference between the vector- and the G3ΔEGF-transfected cells before serum withdrawal. After serum withdrawal, however, the vector- transfected cells rapidly arrested in the G1 phase of the cell cycle, whereas the G3ΔEGF-transfected cells demonstrated a much slower response [see Supplementary Fig. S1 (http://jcs.biologists.org/supplemental/)]. The result is in agreement with the growth curves seen after serum withdrawal (Fig. 3C).

In serum-free cultures, the cells were prepared and seeded on tissue-culture plates in serum-free DMEM. The cultures were therefore totally devoid of nutrients compared with the serum-withdrawal cultures. 24 hours after cell inoculation, the vector-transfected cells developed much longer processes and interacted with each other, whereas the G3ΔEGF-transfected cells were less elongated [see Supplementary Fig. S2 (http://jcs.biologists.org/supplemental/)]. 2 days after cell inoculation, the vector-transfected cells continued to extend processes, whereas most of the G3ΔEGF-transfected cells died. After 8 weeks of culture, the vector-transfected cells had migrated, aggregated and survived, whereas all the G3ΔEGF-transfected cells died within 1 week. Cell counts indicated that half of the vector-transfected cells could be maintained in the absence of serum for many weeks, whereas the G3ΔEGF-transfected cells quickly died and were degraded within 1 week (Fig. 3E). Flow cytometry once again revealed that the G3ΔEGF-transfected cells had a higher rate of apoptosis than the vector-transfected cells (data not shown). It appears that the G3ΔEGF-transfected cells need exogenous nutrients for survival. The vector-transfected cells were able to adjust and adapt to changes of culture conditions. When the nutrient levels were low, these cells stopped growing, changed morphology and survived. These results suggest that G3ΔEGF-transfected cells lose the ability to survive in nutrient-poor conditions.

Analysis of EGFR phosphorylation indicated that the levels of the receptor tyrosine phosphorylation significantly

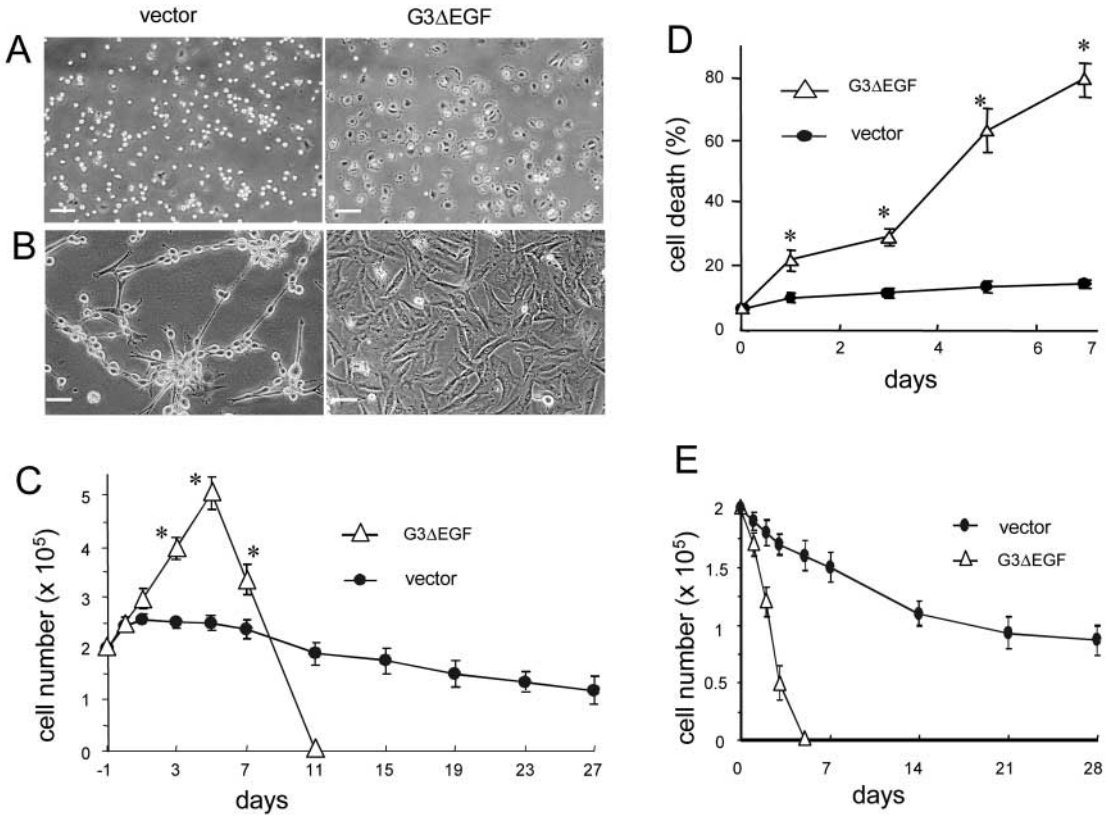

Fig. 3. Effects of G3ΔEGF on cell growth under different culture conditions. (A) Cells (2×10^5 cells per well) were seeded on six-well culture plates maintained in DMEM containing 10% FBS. 1 hour after cell inoculation, G3ΔEGF-transfected U87 cells exhibited spreading on the plates, but vector-transfected U87 cells had only started to attach. Scale bars, 40 μm. (B) Serum was withdrawn from the cultures by changing the culture medium to DMEM alone. At 24 hours, most of the vector-transfected cells had rounded and some had developed long processes to connect to each other, but the G3ΔEGF-transfected cells remained spread on the plates. (C) Cells before and after serum withdrawal were harvested at different time intervals and cell numbers were counted to obtain a growth curve. The total number of the vector-transfected cells declined slowly. However, the G3ΔEGF-transfected cells continued to grow for 5 days, followed by dramatic cell detachment and cell death. (D) Cell death was determined by trypan-blue staining. G3ΔEGF transfection enhanced cell death after serum withdrawal. (E) Cultured in serum-free DMEM, the vector-transfected cells could be maintained for 4 weeks, whereas the numbers of G3ΔEGF-transfected cells declined dramatically owing to cell death. All the experiments were repeated four times. *P<0.01.

decreased after serum withdrawal. This effect was more evident in the G3ΔEGF-transfected cells than in the vector-transfected cells (Fig. 4A). The levels of EGFR protein, however, were not affected. The starved cultures were treated with EGF (100 ng ml^{-1}) in the presence or absence of EGFR inhibitor AG1478. The introduction of AG1478 completely abolished EGFR phosphorylation in both the vector- and G3ΔEGF-transfected cells (Fig. 4B). AG1478 treatment inhibited growth of the cells and dampened the growth advantage of the vector-transfected cells over the G3ΔEGF-transfected cells in low-serum culture conditions (Fig. 4C). The addition of AG1478 also increased apoptosis of G3ΔEGF-transfected cells from 39% to 52% (a 33% increase), and increased apoptosis of vector-transfected cells from 13% to 45% (a ~250% increase) (Fig. 4D). These results indicate that the EGFR inhibitor AG1478 had greater effects on vector-transfected cells than on G3ΔEGF-transfected cells.

Effect of G3ΔEGF is modulated by EGF

To examine the involvement of EGF in G3ΔEGF-modulated cell behavioral changes, vector- and G3ΔEGF-transfected U87 cells were cultured overnight in DMEM containing 10% FBS, followed by serum withdrawal and the addition of 10 ng ml^{-1} EGF. Treatment with EGF caused different responses between the vector- and G3ΔEGF-transfected cells. EGF promoted cell rounding in vector-transfected cells, but this effect was less evident in G3ΔEGF-transfected cells [see Supplementary Fig. S3 (http://jcs.biologists.org/supplemental/)]. This is in agreement with the effect of EGF on reducing cell spreading (Cao et al., 2000; Lu et al., 2001). The addition of 40 ng ml^{-1} EGF after serum withdrawal produced a greater effect on the proliferation of the G3ΔEGF-transfected cells (Fig. 5A) than on that of the vector-transfected cells (Fig. 5B). As a result, the number of G3ΔEGF-transfected cells was much greater. The addition of EGF also greatly reduced the apoptosis rate in the G3ΔEGF-transfected cells (Fig. 5C).

To dissect the different effects of EGF on G3ΔEGF- and vector-transfected U87 cells, EGF was added to the starved cultures at an excess (100 ng ml^{-1}) to saturate the receptors completely. After starvation, the vector-transfected cells maintained a higher EGFR phosphorylation level than the

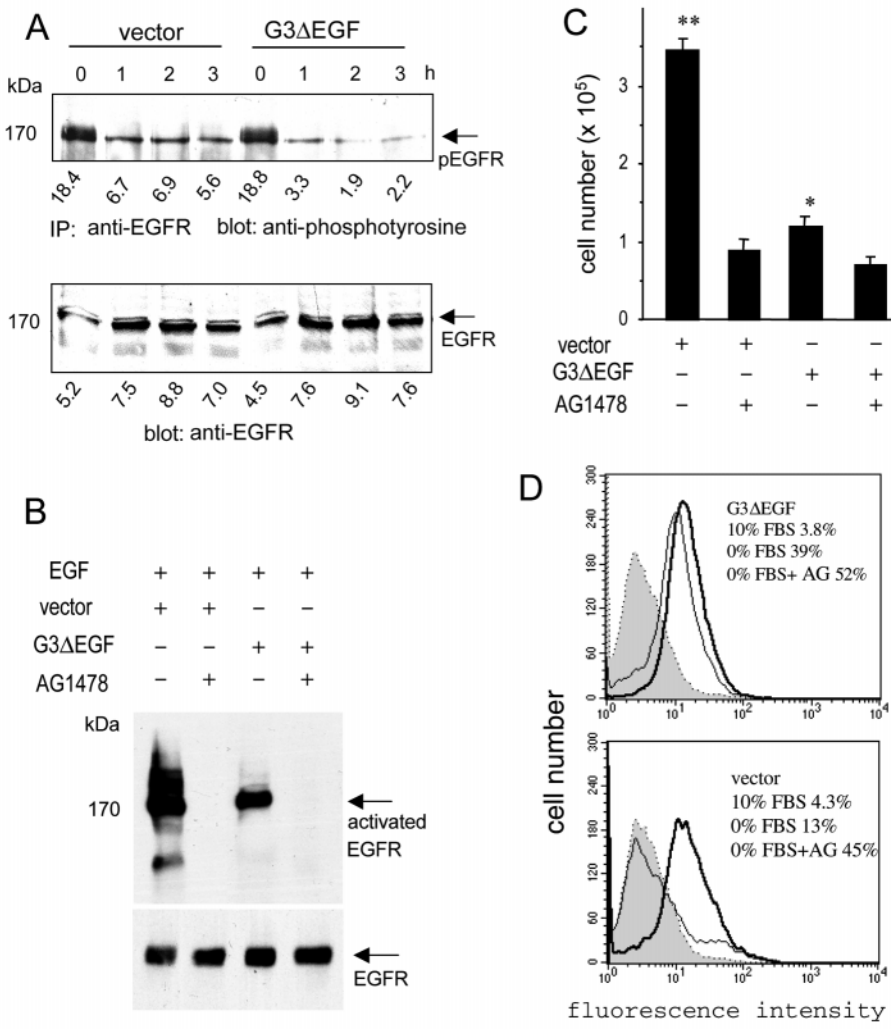

Fig. 4. Involvement of the EGFR in G3ΔEGF functions. (A) Cell lysate was prepared from cultures before and after serum withdrawal and immunoprecipitated with anti-EGFR antibody, followed by western blotting probed with anti-phosphotyrosine antibody. U87 cells expressing G3ΔEGF had lower levels of tyrosine-phosphorylated EGFR (pEGFR) after serum withdrawal, but EGFR expression was similar at different time points. Densitometry readings of protein bands are provided below the blots. (B) Both types of cells were starved and then treated with EGF (100 ng/ml) in the presence or absence of AG1478. EGFR phosphorylation was abolished by AG1478 in both types of cells, whereas the protein levels of EGFR were not affected. (C) Cultured in DMEM containing 1% FBS in the presence or absence of AG1478, the growth-inhibitory effect of AG1478 on vector-transfected cells was much more severe than on G3ΔEGF-transfected cells (AG1478– vs AG1478+: $^{**}P<0.001$; $^{*}P<0.05$). (D) When AG1478 was added to the cultures after serum withdrawal, cell apoptosis was accelerated more obviously in the vector-transfected cells (from 13% to 45% apoptosis, or a ~250% increase) than in G3ΔEGF-transfected cells (from 39% to 52% apoptosis, or a 33% increase). All the experiments were repeated three times.

G3ΔEGF-transfected cells. EGF stimulation caused an increased EGFR phosphorylation, but the phosphorylation levels in the G3ΔEGF-transfected cells were again lower than in the vector-transfected cells (Fig. 5D). The protein levels of EGFR in the G3ΔEGF-transfected cells were much lower than in the vector-transfected cells after EGF stimulation (Fig. 5E). This might be due to increased degradation of EGFR following its phosphorylation.

Expression of G3ΔEGF enhances FAK phosphorylation and EGFR/integrin association

FAK is an important modulator of cell morphology, so we examined whether G3ΔEGF transfection affected FAK phosphorylation. G3ΔEGF- and vector-transfected U87 cells were cultured in DMEM containing 10% FBS overnight, followed by serum withdrawal. Analysis of FAK phosphotyrosine indicated that the cells expressing G3ΔEGF had a higher level of FAK phosphorylation than the vector controls (Fig. 6). Expression levels of FAK and β_1-integrin total protein remained constant at all time points. Consistent with this, G3ΔEGF-transfected cells formed more and larger focal contacts as identified by vinculin and actin staining than the vector-transfected cells before serum withdrawal (Fig. 7). After serum withdrawal, focal contacts could still be detected in the G3ΔEGF-transfected cells but not in the vector-transfected cells.

We investigated the possible association between cell growth and the interaction of EGFR and integrin. Integrin has been shown to interact with G3ΔEGF and to mediate cell adhesion (Wu et al., 2002). Vector-transfected cells were cultured in DMEM containing 0% or 10% FBS overnight. In the presence of serum, β_1-integrin was precipitated with EGFR (Fig. 8A). This suggested that cell growth was associated with the binding of EGFR to β_1-integrin. We also investigated this in a different cell system, Jurkat T-cells. The cells were cultured in DMEM containing 10% FBS for 4 days. Cells in suspension (80% of total cells) were pelleted and lysed. Cells attaching to the plates (20% of total cells) were lysed directly by using the same volume of lysis buffer as in the suspension. Cell lysate was precipitated with anti-EGFR antibody, followed by western blotting probed with anti-integrin antibody. Even at lower protein levels (20% vs 80% nonadherent), the adherent culture had a higher level of integrin-EGFR interaction (Fig. 8B). Cell cycle analysis indicated that greater proportions of cells in the adhesion cultures were detected in the S phase, implying higher levels of proliferation (Fig. 8C).

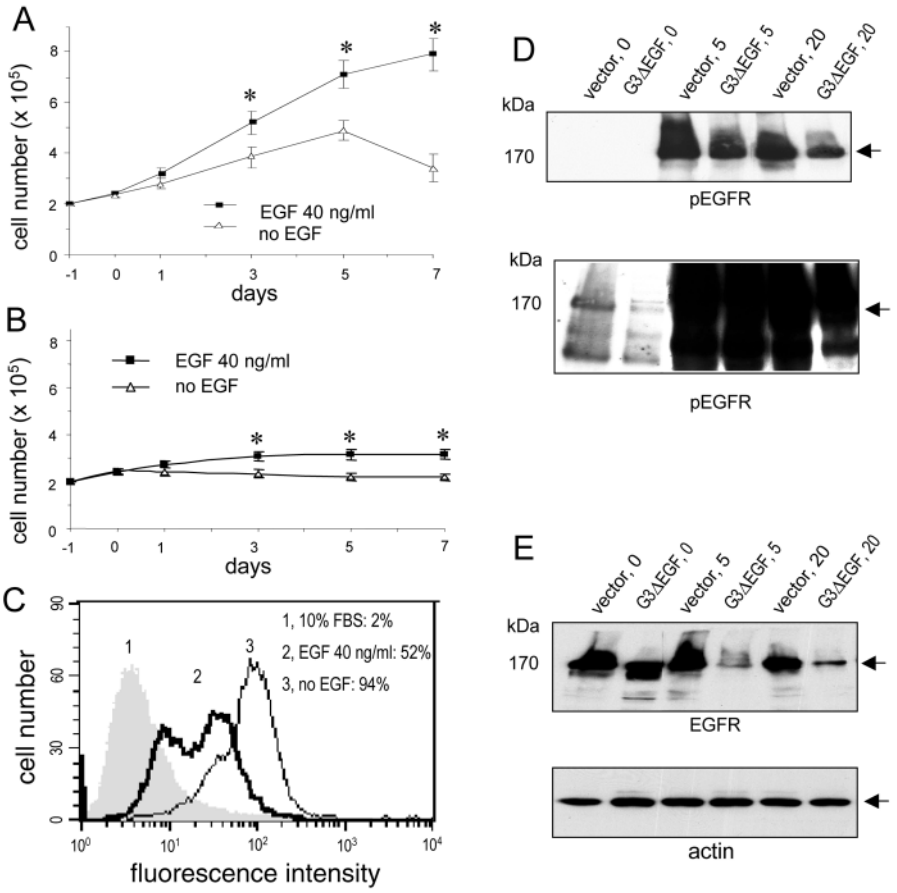

Fig. 5. Effects of EGF on cell proliferation, apoptosis and EGFR phosphorylation. After serum withdrawal, cultures were maintained in the presence or absence of EGF (40 ng ml^{-1}). Cell numbers were counted at days –1 (cell inoculation in DMEM containing 10% FBS), 0 (serum withdrawal and EGF addition), 1, 3, 5 and 7. The addition of EGF had a greater effect on the growth of G3ΔEGF-transfected U87 cells (A) than vector-transfected U87 cells (B). Data are expressed as the means±SD of three experiments each performed in triplicate. *P<0.01. (C) Cell apoptosis was analysed in the G3ΔEGF-transfected cells. After serum withdrawal, the addition of EGF reduced cell apoptosis to 52% on day 7 compared with cells maintained in the absence of EGF (94% undergoing apoptosis). Cells cultured in DMEM/10% FBS were used as controls for the analyses (n=3). (D) After starvation, the cultures were treated with EGF (100 ng ml^{-1}) for 0, 5 or 20 minutes, as indicated. Cell lysate was prepared for western blotting probed with anti-phosphorylated-EGFR antibody. (Top) 1 minute exposure; (bottom) 30 minutes exposure. (E) The same amount of cell lysate was also analysed and probed with anti-EGFR antibody. G3ΔEGF expression enhanced EGFR turnover (arrow). The same membrane was also probed with anti-actin antibody (arrow) to ensure equal loading of protein samples. Results presented in (D) and (E) were repeated twice.

Finally, the effect of G3ΔEGF on EGFR/integrin interaction was investigated. G3ΔEGF- and vector-transfected U87 cells were cultured in DMEM containing 10% FBS overnight, followed by serum withdrawal. Cell lysate was prepared and co-immunoprecipitated with anti-EGFR antibody at different time intervals, followed by western blot analysis probed with anti-β1-integrin antibody. The cells expressing G3ΔEGF exhibited higher levels of EGFR/β1-integrin association (Fig. 8D). After serum was deprived from the cultures, EGFR and integrin rapidly disassociated in the vector-transfected cells, whereas this response was much delayed in the G3ΔEGF-transfected cells.

Discussion

In the ECM, versican is known to associate with several molecules (Aspberg et al., 1995; Isogai et al., 2002; Kawashima et al., 2000; LeBaron et al., 1992; Matsumoto et al., 2003; Yamagata et al., 1986). It is only expressed in tissues where cells are metabolically active and proliferating (Zhang et al., 2001; Zimmermann et al., 1994) and is highly expressed in tumors such as breast and brain tumors (Nara et al., 1997; Paulus et al., 1996). In our studies, we have demonstrated that versican promotes cell proliferation (Yang et al., 2003; Zhang et al., 1999; Zhang et al., 1998b). However, transfection of a mutant construct containing the C-terminal domain of versican (G3ΔEGF) exerted a dominant-negative effect on cell proliferation by inhibiting the secretion and binding of endogenous versican (Wu et al., 2001). To understand how G3ΔEGF affects cellular activities, we have examined cell behavior in the astrocytoma cell line U87 stably transfected with G3ΔEGF. We observed that G3ΔEGF transfection was associated with a loss of both growth contact inhibition and tumor phenotype. Tumorigenic studies indicated that the G3ΔEGF-transfected cells also lost the ability to form tumors, suggesting that versican can play a role in tumor formation.

Under normal growth conditions, both G3ΔEGF- and vector-transfected cells proliferated well but demonstrated differences in cell adhesion, morphology and growth rate. When serum was withdrawn, however, G3ΔEGF-expressing cells continued to proliferate for a short time, followed by massive apoptosis. By contrast, the vector-transfected cells were able to adjust their proliferation rate, adherence and morphology in response to the loss of serum. These cells quickly detached, rounded, migrated, aggregated, formed spheroid-like structures and survived for up to several months. After serum withdrawal, the vector-transfected cells were immediately arrested in the G1 phase, whereas a much greater proportion of G3ΔEGF-expressing cells retained cell cycle progression. Arrest in G1 phase and resistance to apoptosis are important features for tumor cells to survive hostile environments and to evade anticancer-agent- and radiation-therapy-induced cell death (St Croix and Kerbel, 1997). Cell-cell interactions are known to protect cells from apoptosis and to cause chemoresistance in solid tumors.

Recently, we have also demonstrated that overexpression of

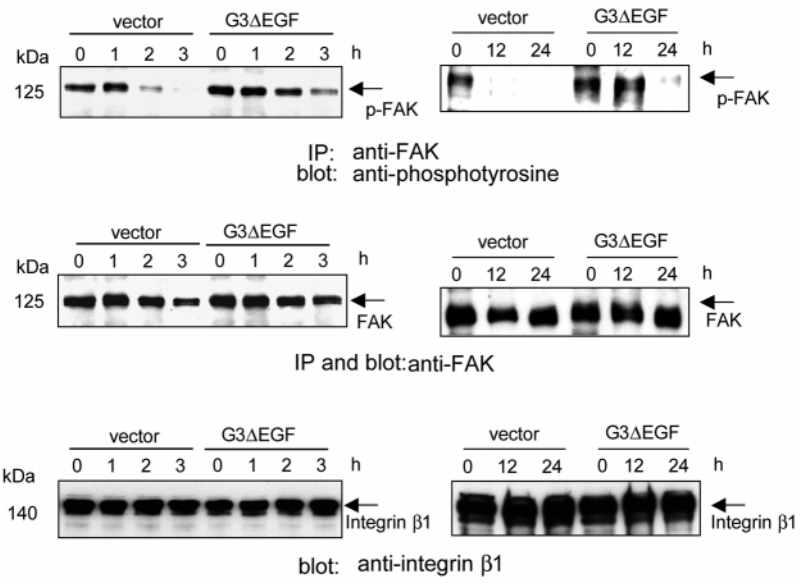

Fig. 6. G3ΔEGF expression increased and maintained FAK phosphorylation. Cells were cultured in DMEM containing 10% FBS for 24 hours followed by serum withdrawal and incubation in serum-free DMEM for different times. The cell lysate was prepared and immunoprecipitated with anti-FAK antibody, followed by western blotting probed with anti-phosphotyrosine antibody. U87 cells expressing G3ΔEGF retained a prolonged FAK phosphorylation (p-FAK) compared with the vector-transfected cells. The same blot was also probed with anti-FAK antibody to assess equal protein loading. Additionally, the cell lysate was analysed on a western blot probed with anti-β_1-integrin antibody. These experiments were repeated three times.

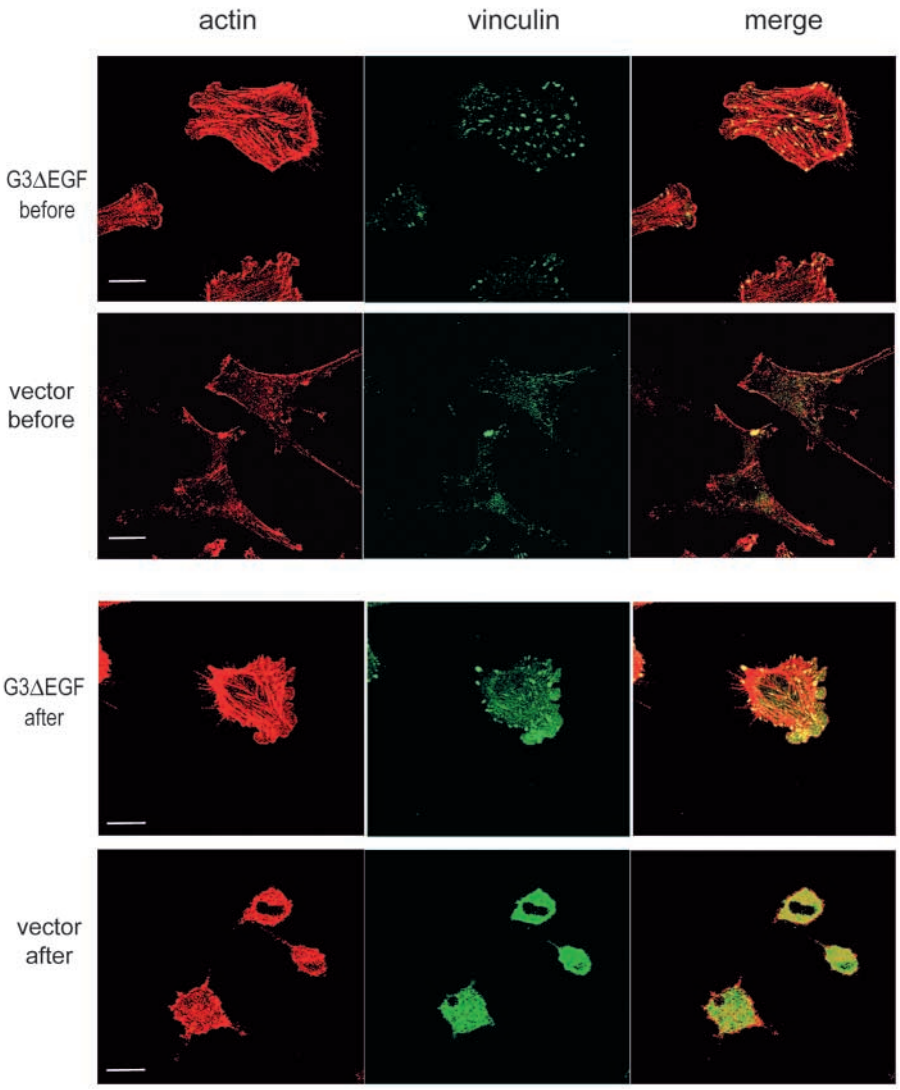

Fig. 7. G3ΔEGF expression enhanced formation of focal contacts. Cells before and after serum withdrawal were fixed and immunostained with TRITC-labeled phalloidin (which binds to actin filaments) and anti-vinculin antibody. G3ΔEGF-transfected U87 cells formed more and larger focal contacts (stained in yellow) than did vector-transfected U87 cells. After serum withdrawal, some focal contacts could still be detected in G3ΔEGF-transfected cells but not in vector-transfected cells. The experiments were repeated three times and one representative result is shown. Scale bars, 20 μm.

a versican G3 construct enhances tumor growth and angiogenesis (Zheng et al., 2004). In this study, we show that transfection of the G3ΔEGF construct inhibits tumor formation. The mechanism by which G3ΔEGF-expressing U87 cells were prevented from forming tumors seems to be associated with the construct's inhibition of endogenous versican function. We have previously demonstrated that overexpression of G3ΔEGF exerts a dominant-negative effect on cell proliferation through the inhibition of endogenous versican secretion and cell surface binding (Wu et al., 2001). Our current results show that astrocytoma cells expressing G3ΔEGF develop an inability to form spheroid-like structures, further suggesting that versican might play a role in enhancing tumor cell-cell interaction and spheroid formation. Lack of the spheroid-like structure might contribute to the reduction of cell survival and loss of tumorigenicity in G3ΔEGF-transfected astrocytoma cells.

Cell transformation is often associated with reduced cell adhesion to the substrate and the uncoupling of cell-cycle dependence on signals that are transduced by integrin-mediated adhesion (Guadagno et al., 1993). In a previous study, we found that G3ΔEGF expression in astrocytoma cells increased β_1-integrin expression and cell adhesion, as well as reducing cell

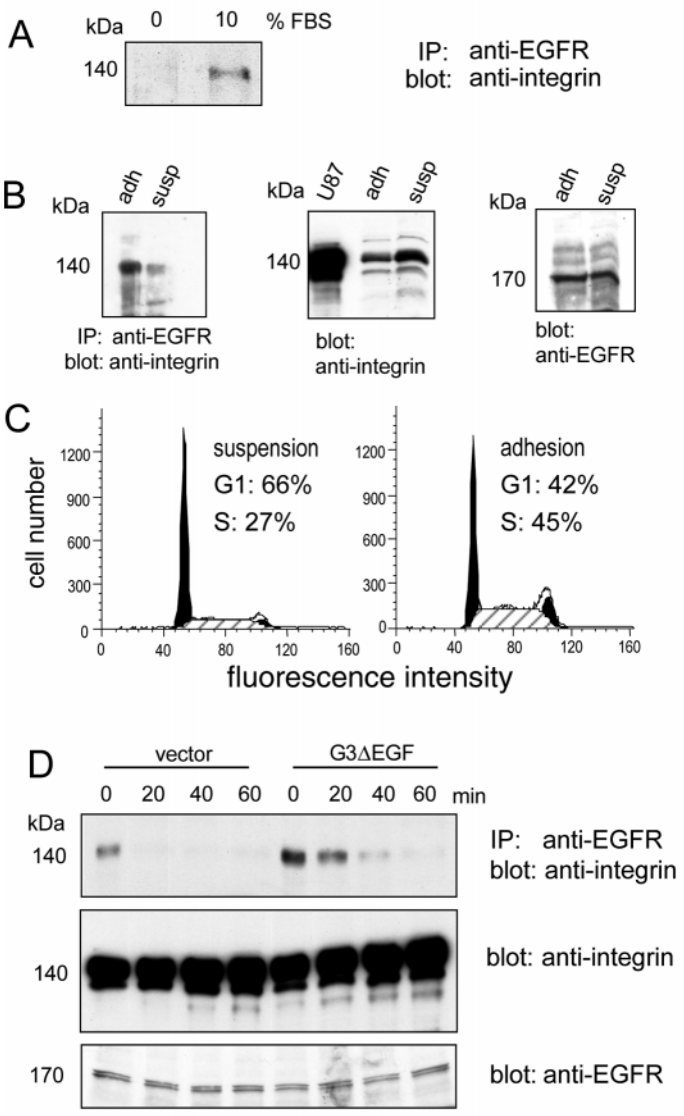

Fig. 8. G3ΔEGF expression promoted EGFR/integrin association. (A) Maintained in different conditions as indicated, U87 cells were lysed and subjected to immunoprecipitation with anti-EGFR antibody, followed by western blot analysis using an anti-β_1-integrin antibody as probe. In the presence of serum, β_1 integrin was precipitated with EGFR. (B) Jurkat cells were cultured in DMEM containing 10% FBS for 4 days. Approximately 20% of the cells attached to the tissue culture plates (adh) and 80% remained in suspension (susp). Both populations of cells were lysed separately with equal amounts of lysis buffer and subjected to immunoprecipitation with anti-EGFR antibody followed by western blot analysis, probed with anti-β_1-integrin antibody. Cell lysate was also analysed by western blotting probed with antibodies against β_1-integrin or EGFR to assess their individual protein levels. Even with lower integrin protein levels (middle), the adherent cultures had higher levels of integrin-EGFR interaction (left). (C) Cell cycles were analysed on the two populations of Jurkat cells. The adherent cells had a larger proportion of cells entering into the S phase (45%) than those in suspension, implying a greater rate of proliferation. (D) G3ΔEGF- and vector-transfected U87 cells were maintained in DMEM containing 10% FBS for 24 hours, followed by serum withdrawal and incubation in serum-free DMEM for different times. Cell lysate was prepared and subjected to immunoprecipitation with anti-EGFR antibody, followed by western blotting probed with anti-β_1-integrin antibody. Expression of G3ΔEGF enhanced the association of EGFR and β_1-integrin before serum withdrawal and delayed the disassociation of EGFR and β_1-integrin after serum withdrawal. Cell lysate was also analysed by western blotting probed with anti-β_1-integrin antibody or anti-EGFR antibody to assess the expression of these two molecules. All experiments were repeated at least three times.

proliferation. In addition, this fragment of versican interacted with β_1-integrin and mediated enhanced FAK phosphorylation (Wu et al., 2002). In the current study, we found that astrocytoma cells expressing G3ΔEGF did not grow in soft agarose gel, whereas the vector-transfected cells formed colonies. This indicates that G3ΔEGF-expressing cells requires secure anchorage to initiate the growth signal. It also suggests that G3ΔEGF-mediated increases in β_1-integrin expression and cell adhesion suppresses tumorigenicity in these cells.

When the culture was deprived of serum, the vector-transfected cells responded by cell cycle arrest, aggregation and survival, whereas the G3ΔEGF-expressing cells maintained adhesion, continued cell cycle progression and eventually underwent apoptosis. This could have been caused by the inhibition of endogenous versican secretion and cell surface binding by G3ΔEGF transfection (Wu et al., 2001), although determining which molecular aspect of versican function is responsible for this effect awaits further experimentation. Nevertheless, the interaction of G3ΔEGF with β_1-integrin (Wu et al., 2002) and the enhanced integrin-EGFR association caused by G3ΔEGF transfection seem to suggest that β_1-integrin/EGFR signal pathway was responsible for much of G3ΔEGF's effect. In addition, we found that integrin and EGFR association correlated with cell proliferation in both astrocytoma and Jurkat cells when both integrin and EGFR were properly activated.

It is known that the collaborative activation of integrin and EGFR leads to enhanced downstream signaling and cell proliferation (Yamada and Even-Ram, 2002). Adhesion-activated integrins have also been shown to interact with EGFR and to phosphorylate it independently of EGF binding (Moro et al., 1998). However, in the present study, EGFR phosphorylation levels were lower in G3ΔEGF-expressing cells than in vector-transfected cells, probably owing to reduced EGF-induced EGFR autophosphorylation. The EGF-like motifs in the G3 domain of versican have been shown to possess growth-factor-like activity in cell proliferation and differentiation, and EGFR is directly involved in these processes (Zhang et al., 1998a; Zhang et al., 1998b). The growth-factor activities of EGF repeats have also been observed in laminin-5 and tenascin-C (Schenk et al., 2003; Swindle et al., 2001). Because expression of the EGF-repeat-containing matrix is tissue specific, further studies will be required to understand how the EGF-harboring matrix functions during development and pathology.

It is not clear whether integrin-induced EGFR phosphorylation differs from EGF-induced EGFR autophosphorylation in mediating cell proliferation and survival, although EGFR autophosphorylation has been known to play an important role in promoting cell survival (Bruns et al., 2000). With insufficient EGF-induced EGFR activation, integrin-initiated growth signals lead to cell cycle progression, but the

cells also increased apoptosis. This notion was supported by further experiments in which EGF was added to the culture, where it significantly reduced apoptosis. It has been reported that phosphorylation of FAK by integrin activation prevented EGF-induced FAK dephosphorylation and inhibited cell migration (Lu et al., 2001). Our study suggests that EGFR-autophosphorylation-mediated FAK dephosphorylation might not only be important for cell rounding and migration but might also be required for cell cycle arrest and cell survival. It has been shown that cells undergo apoptosis when they receive confusing growth-regulatory signals in which both 'go' and 'stop' signals are triggered simultaneously (Prendergast, 1999). In the presence of an insufficient amount of EGF, then, the adhesion-mediated growth signal might mislead the cells to cell cycle progression and cause apoptosis in G3ΔEGF-transfected cells, as in the case of c-Myc-induced apoptosis (Prendergast, 1999).

In conclusion, our studies have suggested a crucial role for the versican C-terminal domain in regulating cell survival and tumorigenesis. Expression of G3ΔEGF significantly altered cell behavior and the cells exhibited anchorage-dependent growth and survival. When exogenous growth factors were depleted through serum deprivation, the G3ΔEGF-expressing cells showed a significantly different response than did vector-transfected cells. These cells demonstrated disturbed cell-cycle regulation and increased apoptosis, increased FAK phosphorylation and reduced EGFR phosphorylation, and increased cell adhesion but reduced cell rounding. All of these effects combine to increase the vulnerability of G3ΔEGF-expressing cells to environmental stress and to abolish G3ΔEGF-expressing cells' capacity for transformation. However, it is not clear whether G3ΔEGF functions through direct interactions with the EGFR-integrin complex or whether the G3ΔEGF product instead acts to modulate the activity of intracellular components associated with these receptors. Further studies will be required to understand this mechanism.

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