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Alteration of collagen-dependent adhesion, motility, and morphogenesis by the expression of antisense α_2 integrin mRNA in mammary cells

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SUMMARY

Although integrins are known to mediate adhesive binding of cells to the extracellular matrix, their role in mediating cellular growth, morphology, and differentiation is less clear. To determine more directly the role of the $\alpha_2\beta_1$ integrin, a collagen and laminin receptor, in mediating the collagen-dependent differentiation of mammary cells, we reduced expression of the integrin by the well differentiated human breast carcinoma cell line, T47D, by stably expressing α_2 integrin antisense mRNA. Flow cytometry demonstrated that the antisense-expressing clones had levels of $\alpha_2\beta_1$ integrin on their surfaces that were decreased by 30-70%. Adhesion of antisense-expressing clones to both collagens I and IV was decreased relative to controls in a manner that correlated with the level of cell surface $\alpha_2\beta_1$ integrin expression. Adhesion to fibronectin and laminin were not affected. Motility across collagen-coated filters in haptotaxis assays was increased for only those clones that exhibited intermediate levels of adhesion to collagen, suggesting that an intermediate density of cell-surface $\alpha_2\beta_1$ integrin optimally supports cell motility. When cultured in three-dimensional collagen gels, T47D cells organized in a manner suggestive of a glandular epithelium. In contrast, antisense-expressing clones with decreased $\alpha_2\beta_1$ integrin were not able to organize in three-dimensional collagen gels. The growth rate of T47D cells was reduced when the cells were cultured in three-dimensional collagen gels. Unlike adhesion, motility, and morphogenesis, growth rates were unaffected by reduction of $\alpha_2\beta_1$ integrin expression. Our results suggest that adhesive interactions mediated by a critical level of surface $\alpha_2\beta_1$ integrin expression are key determinants of the collagen-dependent morphogenetic capacity of mammary epithelial cells.

Key words: integrin, collagen, morphogenesis, mammary cell, motility, adhesion, antisense

INTRODUCTION

Continuous contact with the extracellular matrix (ECM) is vital for epithelial cells to maintain proper cellular morphology and differentiation-specific gene expression (Bernfield et al., 1984; Stoker et al., 1968; Mauchamp et al., 1987; Watt et al.,1987; Pignatelli and Bodmer, 1988; Barcellos-Hoff et al., 1989; Blum et al., 1989; DiPersio et al., 1991; Liu et al., 1991; Dunn et al., 1992). Interactions with the ECM are altered when epithelial cells become malignant, as they no longer remain attached and stationary on ECM. The loss of normal interaction with the ECM is associated with a loss of cellular differentiation and adhesion-dependent regulation of cell growth (Stoker et al., 1968). Despite considerable literature describing the effects of the ECM on both normal and malignant cellular phenotype, the molecular mechanisms by which the ECM affects cellular phenotype are not clear.

Cells interact with the ECM through a variety of cell-surface receptors, including members of the integrin family (for review see Hynes, 1992; Sonnenberg, 1993). Integrins are transmembrane heterodimers composed of an alpha and a beta subunit. Currently, at least 14 alpha subunits and 9 beta subunits are known (Sonnenberg, 1993), the particular combination of which determines ligand specificity. Many integrins bind to multiple ECM components, and most cells express multiple integrins on their surface. This diversity allows cells to modulate their response to the complex extracellular environment. Integrins are now known to be involved in transducing signals to the cell (for review, see Juliano, 1994), indicating that they mediate more than just adhesion to the ECM.

Recently, attention has turned to defining the role of integrins in mediating the effects of the ECM on cellular differentiation (Menko and Boettiger, 1987; Werb et al., 1989; Stephens et al., 1993). Many epithelial cells grow in an organized manner and express differentiation-specific genes when cultured in three-dimensional gels composed of collagen or EHS tumor matrix (Matrigel), but lose this differentiated phenotype when cultured on plastic surfaces (Pignatelli and Bodmer, 1988; Barcellos-Hoff et al., 1989; Blum et al., 1989; DiPersio et al., 1991; Liu et al., 1991; Streuli et al., 1991; Dunn et al., 1992; Schmidhauser et al., 1990; Matter and Laurie, 1994). A role for integrins has been implied in a few of these systems, such as the in vitro formation of kidney tubules (Sorokin et al., 1990), capillaries (Gamble et al., 1993) and

lung branches (Roman et al., 1991), and the contraction of collagen gels (Klein et al., 1991; Schiro et al., 1991).

The potential role of integrins in regulating differentiation is also implied by alterations in integrin levels during neoplastic transformation (for review, see Dedhar, 1990; Albelda, 1993; Juliano, 1994). In addition to several correlative studies (Plantefaber and Hynes, 1989; Zutter et al., 1990; Koukoulis et al., 1991; Pignatelli et al., 1992), a handful of in vitro transfection studies have more definitively established a role for integrins in mediating the differentiated or transformed state of a cell. Chan et al. (1991) expressed the human α_2 integrin on rhabdomyosarcoma cells and found that it conferred an invasive capability (Chan et al., 1991). Since neither normal skeletal muscle nor rhabdomyosarcomas (skeletal muscle cell tumors) express the $\alpha_2\beta_1$ integrin (Mechtersheimer et al., 1994), the results, while extremely interesting, may not mimic those of actual tumors that arise in vivo. In two other studies, the expression of either the α_5 integrin subunit on CHO cells (Giancotti and Ruoslahti, 1990) or the expression of the α_4 integrin subunit on melanoma cells (Quian et al., 1994) caused the cells to become less motile and invasive. In the case of α_4 integrin expression, the anti-tumorigenic effect was actually due to an increase in homotypic cell-cell adhesion (Quian et al., 1994). Similarly, decreasing cell-cell adhesive events by modulating cell-surface E-cadherin levels causes MDCK and NMuMG cells to become more invasive (Vleminckx et al., 1991).

Mammary cells acquire a glandular morphology, synthesize an organized basement membrane, and maintain mammary-specific gene expression when cultured in Matrigel (Barcellos-Hoff et al., 1989; Blum et al., 1989; Streuli et al., 1991; Schmidhauser, 1990). However, studies of the role of integrins in mammary differentiation have been limited, since most of the functional studies to date have used primary mouse mammary cells, and very few specific function-blocking antibodies against mouse integrins exist. Because of these limitations, Streuli et al. (1991) found that the β_1 integrin family is involved in mammary morphogenesis, but were unable to define a specific alpha subunit. Adult human mammary gland expresses a high level of the $\alpha_2\beta_1$ integrin (Zutter et al., 1990), and two recent studies suggest the $\alpha_2\beta_1$ integrin might mediate some mammary cell responses to collagen. First, the $\alpha_2\beta_1$ integrin mediated the ability of cells to wrap around collagen fibers that were added to an established mammary cell monolayer (Berdichevsky et al., 1992). However, no three-dimensional collagen gel morphogenesis was demonstrated in this study. Second, a change in c-*erb*B2 expression in mammary cells caused a change in $\alpha_2\beta_1$ integrin levels, but it was impossible to know whether the $\alpha_2\beta_1$ integrin had a primary effect on collagen morphogenesis that was separate from c-*erb*B2 expression (D'Souza et al., 1993). Additional studies are necessary to more clearly define the role of the $\alpha_2\beta_1$ integrin in mediating mammary differentiation and morphogenesis.

Previous work in our laboratory correlated decreased levels of $\alpha_2\beta_1$ integrin expression with decreasing degrees of tumor cell differentiation in breast adenocarcinomas (Zutter et al., 1990). This finding suggested that a loss of α_2 integrin might lead to a decrease in the differentiation state of mammary cells and to an increase in their invasive capability. In order to study this directly in vitro, a series of clonal cell lines was created that have a common genetic background and express varying levels of $\alpha_2\beta_1$ integrin. In this study, we demonstrate the successful use of antisense mRNA to disrupt the cell-surface expression of the $\alpha_2\beta_1$ integrin in a well-differentiated human breast adenocarcinoma cell line, T47D. Decreased $\alpha_2\beta_1$ integrin levels caused a corresponding decrease in cell adhesion to collagen. Interestingly, cell motility on collagen was optimal at an intermediate level of integrin expression. Furthermore, a decrease in $\alpha_2\beta_1$ integrin levels disrupted the ability of mammary cells to organize in three-dimensional collagen gels, clearly demonstrating that $\alpha_2\beta_1$ integrin plays a critical role in collagen gel-induced morphogenesis. Taken together, these results imply that the level of $\alpha_2\beta_1$ integrin expression affects mammary cell differentiation.

MATERIALS AND METHODS

pREP4α2′ construct formation and transfection of T47D cells

A 1.3 kb partial length α_2 integrin cDNA (Burger et al.,1992), representing the first 1289 base pairs of the published cDNA sequence (Takada and Hemler, 1989), and containing the first 353 amino acids and the translation start site, was subcloned into the multiple cloning site of the pREP 4 expression vector (InVitrogen, San Diego, CA) to generate pREP4α2′ (Fig. 1). The pREP4 vector contains an EBV oriP, a hygromycin resistance gene for selection in mammalian cells, and the RSV promoter to drive mRNA transcription. This vector was transfected into an EBNA-expressing T47D cell line. To generate the EBNA positive T47D cell line, cells were co-transfected with pCMV/EBNA and pSV2 neo (both from Clontech, Palo Alto, CA) at a 10:1 ratio of pCMV/EBNA: pSV2neo. Transfectants were selected in 300 μg/ml G418 (Gibco BRL, Grand Island, NY). For all transfections, a 75 cm^2 flask of cells at 70% confluency was transfected with 20 μg of plasmid DNA using the lipofectin reagent (Gibco BRL) for 8 hours. After transfection of pREP4α2′ into EBNA positive T47D cells, transfectants were selected in 125 μg/ml or 250 μg/ml hygromycin B (Calbiochem, San Diego, CA). After one month, small colonies of cells were scraped off the flask using a cloning loop and expanded as clones. Cells were maintained in hygromycin B to avoid loss of the pREP4α2′ episome. To generate the control T-REP4 cell line, EBNA positive T47D cells were transfected as above with the pREP4 vector containing no insert, and selected in 250 μg/ml hygromycin B.

T47D cells were obtained from ATCC (Rockville, MD) and maintained mycoplasma free in RPMI (Gibco BRL) supplemented with 10% fetal bovine serum (Gibco BRL), 0.2 units/ml insulin (Novo Norodisk Pharmaceuticals, Princeton, NJ), 100 ng/ml pennicillin-streptomycin, gentamicin, and 2 mM L-glutamine (Gibco BRL).

Flow cytometry

Cells were prepared for flow cytometry just before analysis. Cells at 60-90% confluency were detached in versene (0.5 mM EDTA in phosphate buffered saline) and washed once in culture medium. For each sample, approximately 0.5×10^6 to 1×10^6 cells were pelleted in a polypropylene tube and resuspended in 0.2 ml ice-cold PBS supplemented with 5 mg/ml BSA containing the appropriate antibody. Cells were incubated with primary antibody at 4°C for 20 minutes, washed with ice-cold PBS/BSA, and resuspended in 0.2 ml secondary antibody. Cells were washed once and maintained on ice until analysis. Cells were analyzed on a Becton-Dickenson FACScan Analyzer using Becton-Dickenson FACSCAN and Lysis software (Becton-Dickenson, Mountain View, CA). Primary antibodies were against integrin subunits as follows: α_2 integrin (P1E6, Gibco BRL); α_5 integrin (P1D6, Gibco BRL); α_1 integrin (TS2/7, a generous gift

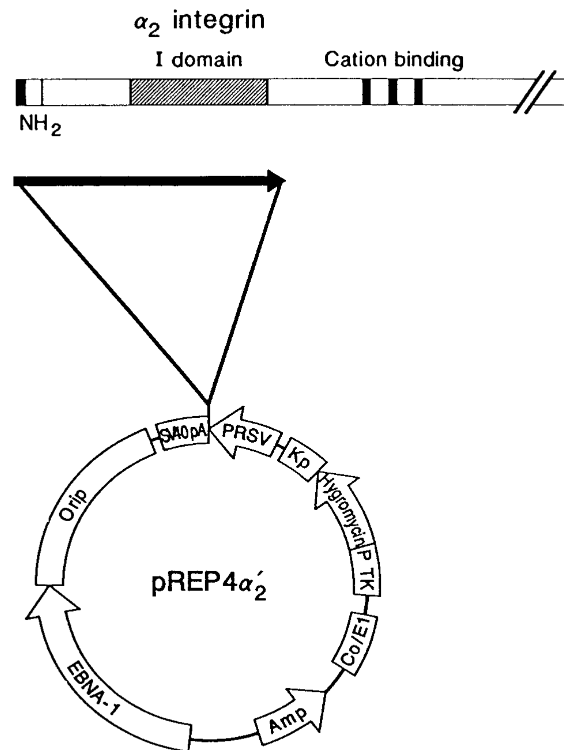

Fig. 1. The pREP4α2′ antisense expression vector construct used to generate antisense-expressing clones. A partial length α_2 integrin subunit cDNA (filled arrow), representing the first 353 amino acids and nucleotides 1-1289 of the published α_2 cDNA sequence was cloned into the pREP4 vector (InVitrogen) downstream from the RSV promoter. A schematic of the functional domains of the α_2 integrin is also shown to denote the portion of the α_2 integrin used to make the antisense construct.

from Dr Martin Hemler); α_3 integrin (P1B5, Gibco BRL); and β_1 integrin (4B4, Coulter Immunology, Hialeah, FL). The secondary antibody was FITC-goat anti-mouse at 10 µg/ml (TAGO Immunologicals, Burlingame, CA).

DNA analysis

Total cellular DNA for Southern analysis was prepared by proteinase K digestion and subsequent phenol/chloroform extraction and ethanol precipitation as described by Davis et al. (1986). RNA was removed by digestion with 50 µg/ml RNase A.

From each sample, 20 µg of DNA was digested with *Eco*RI and electrophoresed on a 0.8% agarose gel. The DNA on the gel was denatured, neutralized, and transferred onto nitrocellulose (Schleicher and Schuell, Keene, NH) in 10× SSC overnight. The blot was baked, then blocked in hybridization solution composed of 50% formamide, 200 mM PIPES, 800 mM NaCl, 0.1% SDS, and 20 mg/ml blocking reagent (Boehringer Mannheim, Indianapolis, IN). A ^{32}P-probe for a 1.8 kb *Eco*RI fragment of the pREP4 vector that includes part of the hygromycin resistance gene was labelled by random priming (using Prime-it II, Stratagene, La Jolla, CA), added to the hybridization solution at 5×10^6 dpm/ml, and incubated at 42°C overnight. The hybridized blots were washed at 60°C in 0.1× SSC/0.1% SDS and exposed to film for 1-2 days.

RNA analysis

Total cellular RNA was prepared by guanidine thiocyanate extraction and preparative ultracentrifugation, as described (Davis et al., 1986). Northern blot analysis was performed by electrophoretic separation of total cellular RNA (15 µg) in denaturing formaldeyde-agarose gels, transfer of the RNA to nitrocellulose membrane (Schleicher and Schull) and baking and hybridizing overnight to ^{32}P random-primed cDNA probes. The hybridized blots were washed at 50°C in 0.1× SSC/0.1% SDS and exposed to film for 1-5 days.

An α_2 integrin probe was made from the same partial-length 1289 bp cDNA clone (Burger et al., 1992) used to generate the pREP4α2′ construct. A GAPDH probe was obtained from ATCC (Rockville, MD).

Adhesion assay

To prepare substrata, rat tail collagen I (Collaborative Biomedical Products, Bedford, MA) or human placenta collagen IV (Sigma Chemical Co., St Louis, MO) was diluted to the appropriate concentration in distilled water, and adsorbed onto Immulon 2 plates (Dynatech, Chantilly, VA) at 4°C overnight. Similarly, fibronectin (prepared from human plasma as described by Ruoslahti et al., 1982) and laminin (Collaborative Biomedical Products) were diluted in PBS and adsorbed as above. Nonspecific sites were blocked with 5 mg/ml BSA (fatty acid free, ICN Biomedicals, Costa Mesa, CA) for 2-3 hours. Cells at 60-90% confluency were detached in versene (0.5 mM EDTA in PBS), centrifuged briefly, and resuspended in PBS supplemented with 5 mg/ml BSA, 5 mM glucose and 0.3 mM $MgCl_2$. Cells were diluted to a concentration of 50,000 cells/ml, and 100 µl were added to each well. Cells were allowed to attach for 15-30 minutes at 26°C. For adhesion to laminin, cells were allowed to attach for 90 minutes at 37°C. Nonadherent cells were removed by rinsing with PBS three times. Adherent cells were quantitated using an assay for hexosaminidase based on the procedure of Landegren (1984) as described by Haugen et al. (1990).

Collagen gel culture

Three-dimensional collagen I gels were made from rat tail collagen (Collaborative Biomedical Products, Bedford, MA) by adding an equal volume of neutralizing buffer (2× Hanks' balanced salt solution, 50 mM HEPES, pH 7.4) and enough culture medium to make a final gel concentration of 1.3 mg/ml. Immediately, cells were added to the solution at a concentration of 2.5×10^5 to 3×10^5 cells/ml, and the solution was transferred into Petri dishes or wells, and warmed to 37°C. After 30 minutes, when gels had formed, culture medium was added. Hygromycin was omitted from the medium added to collagen gel cultures of antisense transfectants. The edge of the gel was rimmed with a sterile Pasteur pipet, and the dish was shaken gently until the gel was free-floating. Medium was changed every 2-4 days by gently replacing part of the volume. It was necessary to culture cells for 8-10 days before morphological changes were noted.

For antibody inhibition, culture medium containing antibodies was added to the gels to give a final concentration of 1:1000. The antibodies used were inhibitory antibodies against the α_2 integrin subunit (P1E6) or against the α_5 integrin subunit (P1D6, both from Gibco BRL, Grand Island, NY).

Motility assays

Motility assays were performed using a modification of the assay described by Herbst et al. (1988). For motility assays, 12 mm Transwell chambers (Costar, Cambridge, MA) with a 12 µm pore polycarbonate membrane were used. The underside of the polycarbonate membrane was coated by adding 0.6 ml of 37 µg/ml rat tail collagen (Collaborative Biochemical Research) or 30 µg/ml fibronectin to the lower chamber of the Transwell. After 3-4 hours, the lower chamber was rinsed twice with RPMI. Cells were detached in trypsin-EDTA (Gibco BRL), washed in medium, and resuspended in RPMI supplemented with 5 mg/ml BSA. Cells were diluted to 200,000 cells/ml and 0.5 ml of cell suspension was added to the top chamber of the Transwell. RPMI was added to the lower chamber, and the assay was incubated for 20 hours at 37°C. Nonmotile cells were scraped off the top side of the polycarbonate membrane with a

cotton swab. Cells that had migrated to the underside of the membrane were fixed and stained in DiffQuick (Baxter, Miami, FL). The filters were excised from their plastic supports and mounted on glass slides with Permount (Sigma Chemical Co.). Cells were counted across two diameters each on duplicate filters.

Immunohistochemistry

T47D cells that had been cultured in three-dimensional collagen gels were fixed in 10% formalin, dehydrated, embedded in paraffin, and sectioned. Sections were prepared for staining by deparaffinizing in xylene, rehydrating through a graded ethanol series, and soaking in PBS. Non-specific sites were blocked in 5 mg/ml BSA, 1% goat serum in PBS. Sections were stained with the HMFG-2 antibody at 1:200 (AMAC, Inc., Westbrook,ME), which recognizes a mucin component of the human milk fat globule. As a secondary antibody, horseradish peroxidase-conjugated goat anti-mouse antibody at 1:3000 was used (Biorad, Richmond, CA). Immunostaining was detected using diaminobenzidine. Sections were counterstained in methyl green.

Western blots

Cells at 60-90% confluency were lysed in Tris-buffered saline (TBS), 1.0% SDS, 10 mM EDTA for 1 hour. Protein levels were determined by the BCA assay (Pierce, Rockford, IL), and 100 μg total protein/lane electrophoresed on a 10% polyacrylamide gel (37.5:1, acry:bis; Amresco, Solon, OH). The gel was transferred onto Immobilon-P (Millipore, Bedford, MA). The membrane was blocked in 2% dried milk, 0.1% normal goat serum in TBS. Primary polyclonal antibody against a 23 amino acid synthetic peptide representing the C-terminal cytoplasmic tail of α_2 integrin (a generous gift from Richard Hynes and Eugene Marcantonio) was diluted in Blotto plus 0.05% Tween-20 and incubated on the blot overnight (Secondary antibody goat anti-rabbit conjugated to horseradish peroxidase was used at 1:100 (Bio-Rad, Richmond, CA). After extensive washing, blots were visualized using ECL (Amersham, Arlington Heights, IL).

Growth assays

Growth assays were performed in two manners. For the first, cells were diluted to 20,000 cells/ml and 0.5 ml/well was seeded into a 24-well tissue culture dish. Cells were grown on plastic or plastic coated with 67 μg/ml rat tail collagen I (Collaborative Biomedical Products). At one- to two-day intervals for 10 days, cells in triplicate wells were detached in trypsin and counted using a hemocytometer. For the second assay, cells were cultured at 250,000 cells/ml and either embedded in collagen gels made as described above or cultured on plastic coated with 67 μg/ml collagen. The same collagen preparation was used for coating the plastic and forming the gels (Collaborative Biomedical Products). After the first 24 hours, [^{3}H]thymidine (6.7 Ci/mmol; Dupont NEN Research Products, Boston, MA) at 0.2 μCi/well was added to the cultures. Triplicate cultures were harvested onto glass fiber filters every two days. Filters were washed with 30 ml of 95% ethanol, dried, and counted in a scintillation counter. To control for [^{3}H]thymidine that may have been trapped in the collagen gels, mock gels containing no cells were labelled and harvested as well, and their background counts (less than 10% of the total recovered) subtracted from all of the samples. Cultures were fed at two-day intervals with medium containing the appropriate amount of [^{3}H]thymidine.

RESULTS

Generation and characterization of antisense clones

We used antisense mRNA to generate stable clonal cell lines of the well-differentiated human breast adenocarcinoma, T47D, that expressed decreased levels of the $\alpha_2\beta_1$ integrin. Preliminary studies using blocking antibodies had established that the $\alpha_2\beta_1$ integrin was the predominant collagen adhesion receptor on T47D cells (data not shown). We used an episomal system to generate multiple antisense mRNA copies per cell. This system takes advantage of the fact that an extrachromosomal plasmid that contains the Epstein-Barr virus (EBV) origin of replication can be maintained in cell lines expressing the EBV nuclear antigen (EBNA) (Hambor et al., 1988). A partial length α_2 integrin subunit cDNA was cloned into the pREP 4 expression vector to generate pREP4α2′ (Fig. 1). This vector was transfected into a T47D cell line that had previously been transfected with the EBNA gene. As a control, the pREP4 vector alone was also transfected into EBNA-positive T47D cells to generate the T-REP4 cell line.

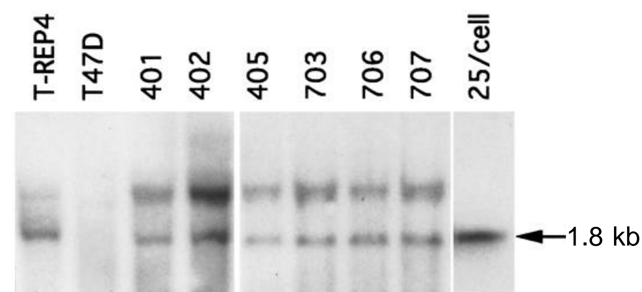

Fig. 2. Southern blot of transfected T47D cells demonstrating the presence of pREP4 vector sequences in the clonal cell lines. Total cellular DNA from T47D cells and transfectants was digested, electrophoresed on an agarose gel, blotted onto nitrocellulose, and hybridized with a probe specific for the pREP4 vector. Note the presence of an expected 1.8 kb vector band in both pREP4-transfected T-REP4 cells and pREP4α2′-transfected antisense clones, 401, 402, 405, 703, 706, and 707. This band is not present in untransfected T47D cells. An amount equivalent to 25 copies/cell of digested pREP4α2′ is shown in the far right lane.

The presence of the pREP4α2′ antisense construct in the selected clones was verified by Southern analysis (Fig. 2). Digestion of total cellular DNA from cells transfected with the pREP4α2′ antisense construct produced an expected 1.8 kb band corresponding to vector sequences. This band was also present in the T-REP4 cell line, but not in the parental T47D cell line. An additional, approximately 3.8 kb sized band that reacted with the vector-specific probe was also noted in most of the clones. This band may represent partially digested DNA, and was also noted in control digests of the pREP4α2′ vector at higher concentrations of DNA. No obvious difference in the amount of vector was noted between the cell lines. On the basis of the intensity of the bands, we estimate that there are between 13 and 17 copies of the pREP4α2′ per cell. We estimate that there are at least this many copies of the pREP4 vector in the control, T-REP4 cell line.

As an initial screen, clones were analyzed for decreased cell-surface $\alpha_2\beta_1$ integrin expression by flow cytometry using the P1E6 antibody against the α_2 integrin subunit. Ten clonal cell lines were generated that had α_2 integrin expression that was decreased approximately 30-70% as compared with the parental T47D cells (examples shown in Fig. 3A). The clones shown are from two different series; the 400-series clones were selected in 125 μg/ml hygromycin, while the 700-series clones were selected in 250 μg/ml hygromycin. By flow cytometry, we could discern three different levels of $\alpha_2\beta_1$ integrin

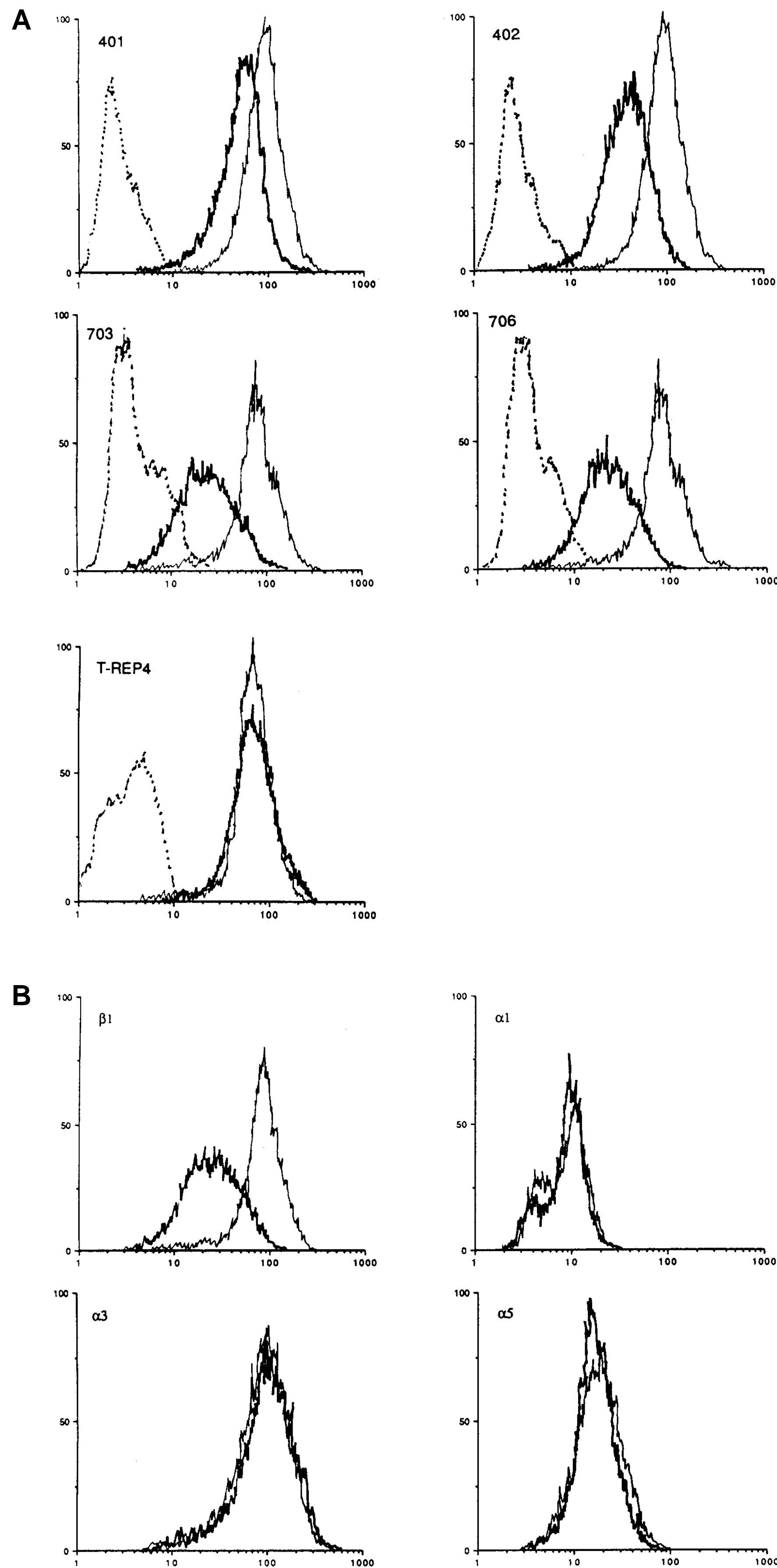

Fig. 3. Flow cytometry of antisense transfectants demonstrating a specific decrease in cell-surface $\alpha_2\beta_1$ integrin expression. (A) Cells were stained with an anti-α_2 integrin antibody (P1E6), shown by the bold trace in each panel. A comparison with α_2 integrin staining for parental T47D cells is shown by the thin trace. The broken trace represents background staining with secondary antibody only, FITC-conjugated goat-anti-mouse. As labelled in the upper left corner of each panel, clones 401, 402, 703 and 706, and control T-REP4 cells are shown. (B) Clone 703, shown by the bold trace, stained with different anti-integrin antibodies: the β_1 integrin (antibody 4B4), the α_1 integrin (TS2/7), the α_3 integrin (P1B5), or the α_5 integrin (P1D6), as labelled in the upper left corner of each panel. A comparison to T47D cells is shown by the thin trace.

expression: that of the control cells (parental), that of the 400-series clones, and that of the 700-series clones. After the initial selection, switching 400-series clones from 125 μg/ml hygromycin to 250 μg/ml hygromycin had no effect on the level of α_2 integrin subunit expressed on the cell surface. The control cell line, T-REP4, transfected with the pREP4 vector alone had no decrease in α_2 integrin levels (Fig. 3A). Identical results were obtained when other anti-α_2 integrin antibodies, 12F1 and P1H5, were used (data not shown). Immunoblots of antisense-expressing clones demonstrated a decrease in α_2 integrin levels similar to that observed by flow cytometry; there were no detectable differences in the molecular mass of the α_2 integrin proteins produced by the antisense-expressing clones (data not shown).

Antisense-expressing clones were also screened for the expression of other integrin subunits. The results shown in Fig. 3B for one clone, 703, are representative of all the clones. Cell-surface levels of the β_1 integrin subunit were decreased by an amount comparable to that of the α_2 integrin subunit (Fig. 3B). This likely reflects the fact that integrin subunits are not known to reach the cell-surface as monomers, but only as $\alpha\beta$ heterodimers (Springer et al., 1984). Additionally, this observation suggests that the $\alpha_2\beta_1$ integrin is a major β_1 integrin on T47D cells, consistent with our previous observation. The effect of the antisense construct was specific for the α_2 integrin, since there was no change in expression of other alpha integrin subunits, α_1, α_3, or α_5 (Fig. 3B).

Even though antisense-expressing clones demonstrated a striking decrease in α_2 integrin protein level, there was no consistently detectable decrease in α_2 integrin mRNA as detected by northern blot analysis (Fig. 4). Interestingly, there were bands that hybridized with the α_2 integrin cDNA probe that were present in antisense clones but not in the control, T-REP4 cell line. The origin of these bands is unknown, and attempts to determine if they represent antisense message using directional riboprobes were unsuccessful. Other investigators have also observed that antisense transcripts have no effect on the endogenous sense mRNA levels, which may indicate that the antisense mRNA acts to block translation by the formation of stable sense-antisense duplexes (see, for example, Iwaki et al., 1994).

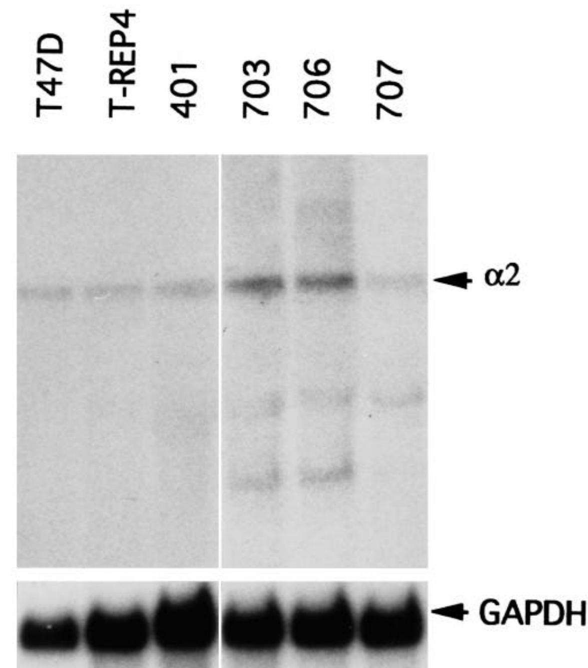

Fig. 4. Northern blot of control, T-REP4, cells and antisense-expressing clones demonstrating the level of α_2 integrin mRNA. Cellular RNA was isolated, electrophoresed on an agarose gel, blotted onto nitrocellulose, and hybridized to a probe for the α_2 integrin. The probe was generated by random-prime labelling the same 1.3 kb piece of the double-stranded α_2 integrin sequence used to generate the antisense construct. The level of the endogenous approximately 8 kb α_2 integrin mRNA (arrow) was not decreased in the antisense-expressing clones. Note that there are novel, α_2 integrin-reactive, bands in lanes containing the antisense clones that are not present in the control, T-REP4 lane. Hybridization to a probe for GAPDH was used to control for RNA loading.

Antisense clones expressing decreased $\alpha_2\beta_1$ integrin have altered adhesion and motility on collagen

To determine the functional consequence of decreasing $\alpha_2\beta_1$ integrin expression, antisense-expressing clones were analyzed for their ability to adhere to collagen substrata. Clones expressing decreased $\alpha_2\beta_1$ integrin exhibited decreased adhesion to both collagens I and IV in a standard adhesion assay (Fig. 5A,B). Control T-REP4 cells adhered to collagen at a level similar to parental T47D cells. Adhesion to collagen I generally paralleled that to collagen IV for a given clone. The adhesion assay delineated subtle functional differences between the clones, and allowed a reproducible grouping of the clones into those whose adhesion to collagen was least affected (402 and 405), intermediately affected (401 and 703) and most affected (706 and 707) relative to controls (T47D and T-REP4). Adhesion assays were also performed to determine whether longer incubation times would allow the adhesion of antisense clones to eventually equal that of control cells. Even after 90 minutes, antisense clones had the same level of decreased adhesion to collagen (not shown). The reduced adhesion of the antisense-expressing clones, as well as the control T-REP4 cells, could be eliminated by function-blocking anti-α_2 integrin antibodies (P1E6, not shown), indicating that the residual adhesion of these clones to collagen is probably due to the remaining $\alpha_2\beta_1$ integrin receptors and not to other collagen receptors.

The antisense-expressing clones showed no decrease in adhesion to fibronectin relative to controls (Fig. 5C). This result is expected, since fibronectin is not a ligand of the $\alpha_2\beta_1$ integrin, but instead of the $\alpha_5\beta_1$ integrin, which was not decreased on the antisense-expressing clones (Fig. 3B). The observed normal adhesion to fibronectin indicates that the effect of the antisense construct on adhesion to collagen is specific, and not due to more general effects on cell adhesion in these clones.

We determined the level of cell adhesion to laminin, which is also a ligand for the $\alpha_2\beta_1$ integrin. Adhesion of T47D cells to laminin in general was not as great as that noted on collagen, and required a much longer incubation. Most of the antisense-expressing clones did not show decreased adhesion to laminin (Fig. 5D). The one exception was clone 706, which showed a slight decrease in adhesion to laminin. The general lack of an

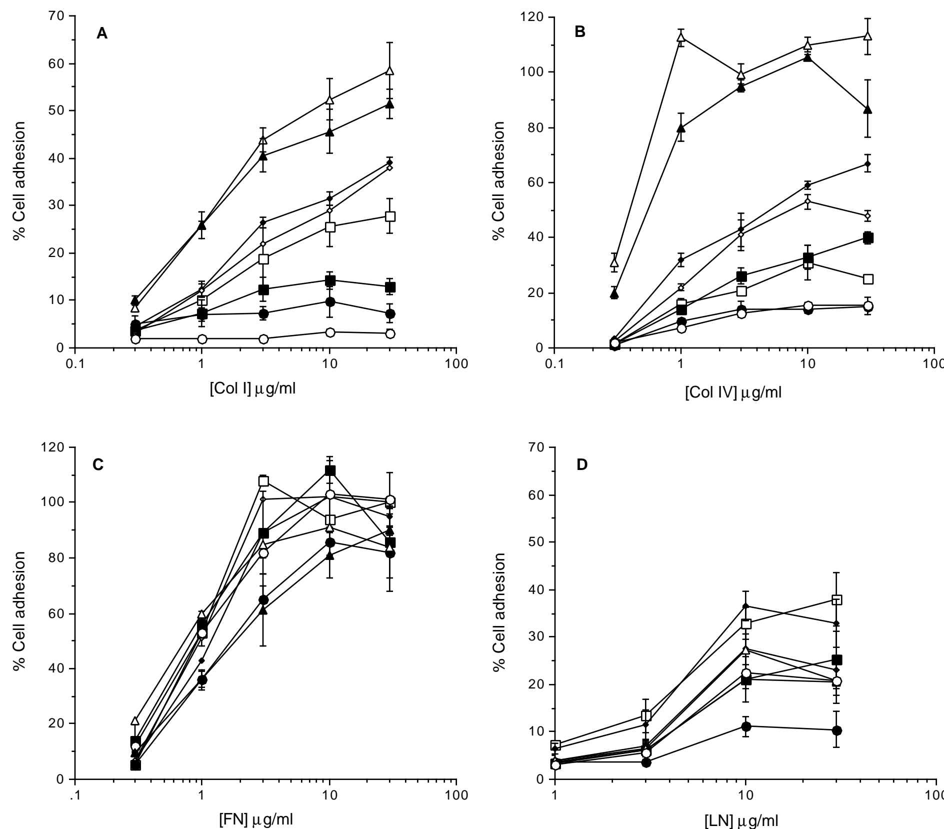

Fig. 5. Clones with decreased $\alpha_2\beta_1$ integrin expression have decreased adhesion to collagens I and IV, but not to fibronectin or laminin. Cells were allowed to adhere to plastic coated with increasing concentrations of: (A) collagen I; (B) collagen IV; (C) fibronectin; or (D) laminin. The concentration of protein added to each well to coat the plastic is indicated on the *x*-axis. Cell lines used were: parent T47D cells (open triangles); control transfected, T-REP4 cells (filled triangles); or antisense-expressing clones: 402 (small filled diamonds); 405 (small open diamonds); 401 (open squares); 703 (filled squares); 706 (filled circles) and 707 (open circles). Each point represents triplicate determinations ± s.d. Values are expressed as a percentage of the total number of cells initially seeded into each well.

effect of decreased $\alpha_2\beta_1$ integrin expression on adhesion to laminin is probably due to the presence of multiple receptors for laminin on the surface of T47D cells, including the $\alpha_3\beta_1$ and $\alpha_6\beta_1$ integrins (Fig. 3B for α_3 and not shown for α_6).

The ability of cells to spread on collagens I and IV, fibronectin, and laminin was also determined. T47D cells spread very slowly, so spreading was determined in a separate assay over several hours. While adhesion of antisense-expressing clones to collagen was reduced, those cells that did adhere to collagen were able to spread comparably to control cells (not shown). Cell spreading on fibronectin or laminin was not affected (not shown).

Cell motility on collagen and fibronectin was determined in in vitro haptotaxis assays (Herbst et al.,1988). Cells were seeded in the top chamber of a Transwell that had been coated from the underside with 30 µg/ml collagen I or fibronectin. After 20 hours, cells that had migrated to the underside of the filter were fixed, stained, and counted. Results from two separate experiments on collagen are shown in Fig. 6A,B. In both experiments, two of the antisense-expressing clones, 401 and 703, demonstrated a marked increase in migration across collagen relative to control, T-REP4 cells (Fig. 6A,B). Clone 405, tested in only one of these assays (Fig. 6A), also exhibited increased motility on collagen. These clones had been classified above as having intermediate decreases in adhesion to collagen relative to control, T47D and T-REP4 cells (Fig. 5A,B). The clones that were the least adherent to collagen, 706 and 707, had no increase in migration across collagen relative to T-REP4 cells. Similar results were obtained for migration across collagen IV (not shown). None of the clones showed an increase in motility across fibronectin (Fig. 6C), indicating that the increase in cell motility was specific for collagen.

$\alpha_2\beta_1$ Integrin mediates the morphological organization of mammary cells in three-dimensional collagen gels

Many cell types have been successfully cultured in three-dimensional gels composed of collagen and found to maintain tissue-specific characteristics and morphological organization. To see if the well-differentiated T47D breast carcinoma cells could organize in collagen gels, they were embedded in collagen I gels and cultured for several days. When grown in three-dimensional collagen gels, T47D cells organized in a manner suggestive of a glandular epithelium. The cells formed round, alveolar-like structures and branching tubules or duct-like structures (Fig. 7B). This morphological organization was very different from the unorganized manner in which T47D cells grew on tissue culture plastic or planar surfaces coated with collagen I (Fig. 7A). Note that growth in collagen gels was associated with a change in cell shape such that the cells become more rounded relative to the flattened morphology of cells on planar collagen surfaces. To further characterize the morphology, cells cultured in collagen gels were embedded in paraffin and sectioned. The tubules appeared to have a lumen that stained with an antibody against a mammary cell marker, the human milk fat globule (HMFG; Fig. 7C).

Interestingly, 8-10 days of culture in the collagen gel were required for the morphological differentiation to occur. The time required to elicit a morphological change could not be shortened by seeding a greater density of cells in the gel, sug-

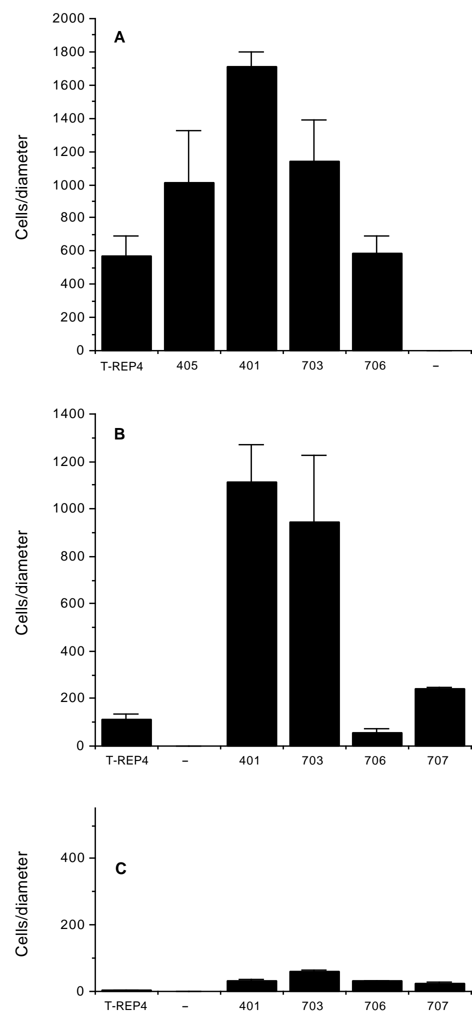

Fig. 6. Cells expressing partially decreased levels of $\alpha_2\beta_1$ integrin are more motile on collagen in a haptotaxis assay. Cells were seeded into the top chamber of a Transwell and allowed to migrate for 20 hours across filters that had been coated from the underside with 30 μg/ml collagen I (A and B) or fibronectin (C). Two different experiments are shown for motility across collagen I. Clones are arranged from greatest adhesion to collagen (left) to least adhesion to collagen (right) and are aligned vertically for direct comparison between experiments. Cells were quantitated by counting across two diameters each of duplicate filters, and are shown ± s.d.

gesting that the lag-time was not due to a requirement for a critical number of cells, but rather to a time-dependent differentiation response of the cells to the collagen gel.

We found no evidence that T47D cells synthesized the basement membrane component, collagen IV, when cultured in collagen I gels. This was determined both by immunofluorescent staining and by in situ hybridization of sections of cells in collagen gels using collagen IV probes. Additionally, Northern blot analysis of RNA from cells cultured in collagen gels did not demonstrate the synthesis of the collagen IV message (data not shown). We also cultured T47D cells in Matrigel, which is composed of basement membrane components. Tubule formation by cells cultured in Matrigel was not as striking as by cells cultured in collagen gels. The cells

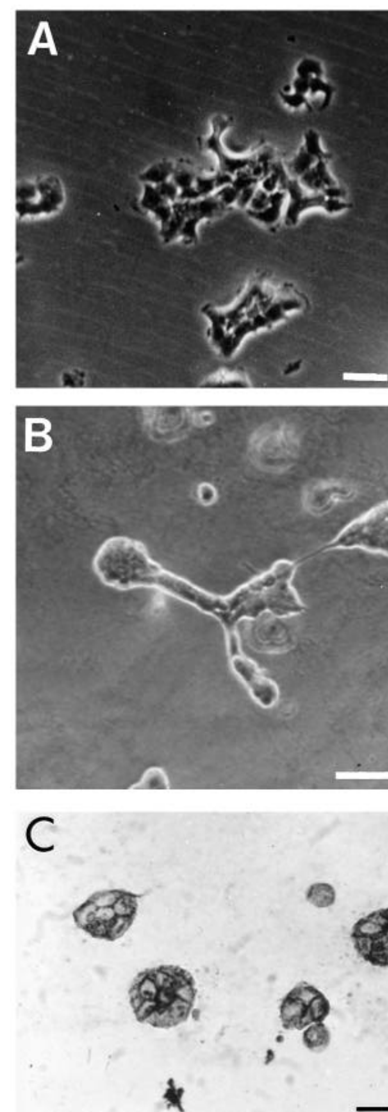

Fig. 7. T47D cells undergo morphological organization when cultured in three-dimensional collagen gels. (A) T47D cells cultured for several days on plastic coated with collagen exhibited a flattened, unorganized morphology. Bar, 50 μm. (B) T47D cells cultured in three-dimensional collagen gels for 10 days formed organized tubule structures that resemble breast ducts. Note the difference in morphology from cells cultured on planar collagen in A. Bar, 100 μm. Both A and B were photographed using phase-contrast microscopy. (C) Cells cultured as in B were paraffin-embedded, sectioned, and stained with an antibody to the human milk fat globule (HMFG-2), which recognizes a breast mucin. Antibody staining was visualized with an HRP-conjugated secondary antibody and diaminobenzadine, and photographed using standard brightfield microscopy. Note the presence of an apparent lumen that was greatly stained with the HMFG-2 antibody. Bar, 30 μm.

formed fewer and less extensive tubules with a larger diameter, or grew as large clumps (data not shown).

The morphological response of T47D cells to collagen gels is mediated by the $\alpha_2\beta_1$ integrin, since α_2 integrin antisense transfectants exhibited disrupted morphology when cultured in

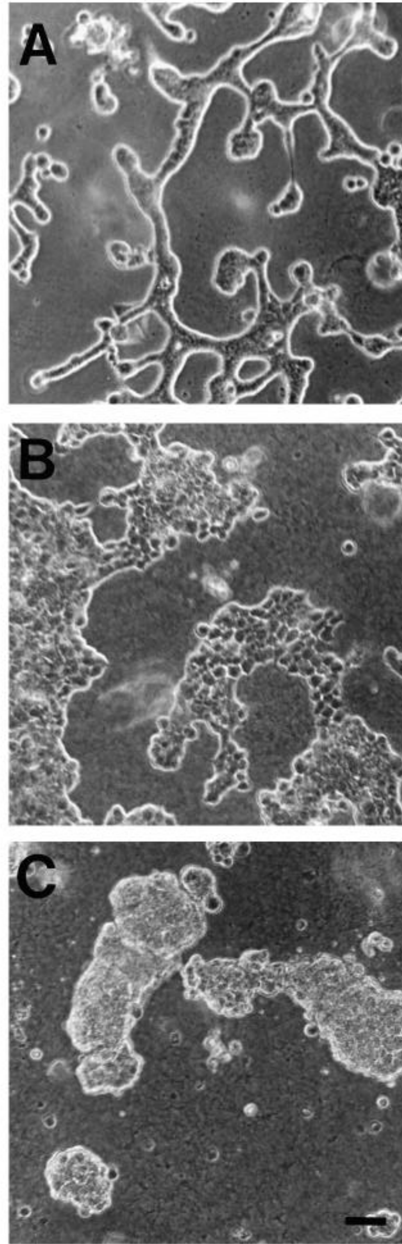

Fig. 8. Antisense-expressing cells with decreased $\alpha_2\beta_1$ integrin levels exhibit disrupted morphology when cultured in three-dimensional collagen gels. Cells were cultured for 10 days in collagen gels, and photographed using phase-contrast microscopy. (A) Control, T-REP4 cells formed tubule structures and organized when cultured in collagen gels. (B) Antisense-expressing clone 401 and (C) antisense-expressing clone 703 did not form tubule structures, but instead grew as disorganized sheets or clumps of cells when cultured in collagen gels. Cells were photographed using phase-contrast microscopy and are all shown at the same magnification. Bar in C, 100 μm.

collagen gels. Control T-REP4 cells formed branching tubules and round, alveolar-like structures when cultured in collagen gels (Fig. 8A). In contrast, antisense-expressing clones did not form any of these structures, but instead grew as disorganized sheets or large clumps of cells (Fig. 8B,C). This striking effect on morphology was observed in both transfectants expressing low as well as intermediate levels of the $\alpha_2\beta_1$ integrin, implying that a critical number of integrins on the surface of the cell is required for the cells to organize in response to collagen gels. The effect was, however, more profound for the low $\alpha_2\beta_1$ integrin expressors, since there were occasional tubule-like structures noted when clones 401 and 405, which are intermediate $\alpha_2\beta_1$ integrin expressors, were cultured in three-dimensional collagen gels. Culture of clones 706 and 707 in collagen gels gave results identical to that shown for clone 703. All of the clones continued to stain for the mammary marker, HMFG (not shown), but since their morphology was disrupted, there was no determination of HMFG localization.

To confirm the role of the α_2 integrin in the morphological organization of cells in three-dimensional collagen gels, cells were also cultured in the presence of function-blocking anti-α_2 integrin antibodies. Anti-α_2 integrin antibodies blocked the ability of T47D cells to form the collagen gel-induced tubule structures. When anti-α_2 integrin antibodies were included in the collagen gel cultures, T47D cells formed disorganized sheets and clumps of cells (Fig. 9B) similar to those seen in cultures of antisense-expressing clones (compare to Fig. 8B,C). Antibodies against the α_5 integrin had no effect on morphology of T47D cells cultured in collagen gels (Fig. 9C).

We observed that culture of T47D cells in three-dimensional collagen gels was associated with a noticeable decrease in the growth rate of the cells. We therefore compared the growth rates of T-REP4 cells and antisense-expressing clones on plastic, on collagen-coated substrata, or in three-dimensional collagen gels by counting cell numbers and labelling cells with [^{3}H]thymidine. Culture on a collagen-coated substratum increased the growth rate of all cell lines relative to culture on plastic (Fig. 10A). There was no detectable difference in growth of the different antisense clones when compared to control, T-REP4 cells. In contrast, cells cultured in three-dimensional collagen gels divided much more slowly than those cultured on planar collagen-coated substrata or on plastic, and seemed to stop growing after 6-8 days in culture (Fig. 10B). Once again, there was little difference in growth rate between T-REP4 cells and antisense-expressing clones, suggesting that regulation of growth rate by collagen gels is probably not mediated through the $\alpha_2\beta_1$ integrin.

DISCUSSION

In this study, we demonstrate that different levels of $\alpha_2\beta_1$ integrin expression on cell surfaces result in altered cellular responses to collagen, and that the $\alpha_2\beta_1$ integrin is critical for collagen-mediated morphogenesis. Our previous studies of human breast carcinomas in vivo revealed a decreased level of $\alpha_2\beta_1$ integrin that correlated with a decreased degree of tumor cell differentiation (Zutter et al., 1990). The results implied that diminished levels of the $\alpha_2\beta_1$ integrin might contribute to a loss of cellular differentiation and an increase in invasiveness. We are now able to extend these correlative studies by demonstrating a direct effect of decreased $\alpha_2\beta_1$ integrin on the differentiation of breast cells, since decreased $\alpha_2\beta_1$ integrin caused T47D cells to be both more motile and less able to organize in collagen gels.

Our results directly address the question of how the density

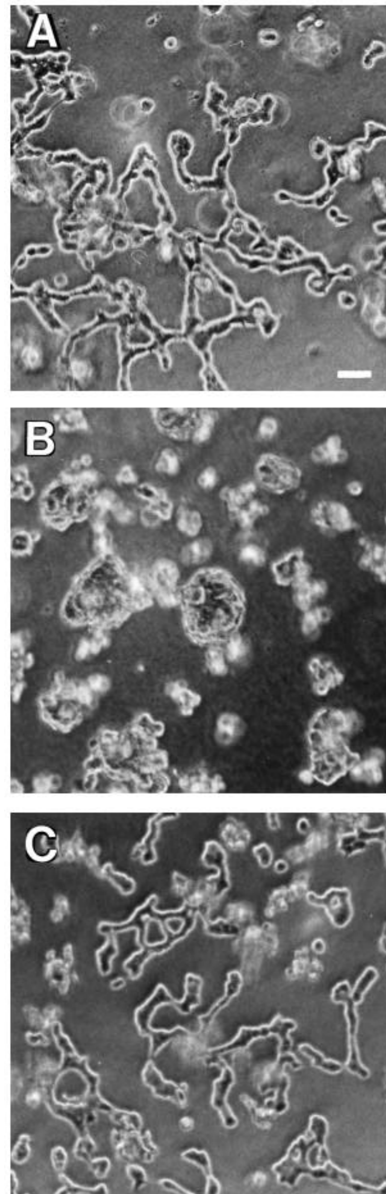

Fig. 9. Anti-α_2 integrin antibodies disrupt the morphological organization of T47D cells cultured in three-dimensional collagen gels. T47D cells were cultured for 10 days in collagen gels to which was added: (A) no antibody; (B) anti-α_2 integrin antibody (P1E6); or (C) control, anti-α_5 integrin antibody (P1D6). Cells were photographed using phase-contrast microscopy and are all shown at the same magnificaton. Bar in A, 100 μm.

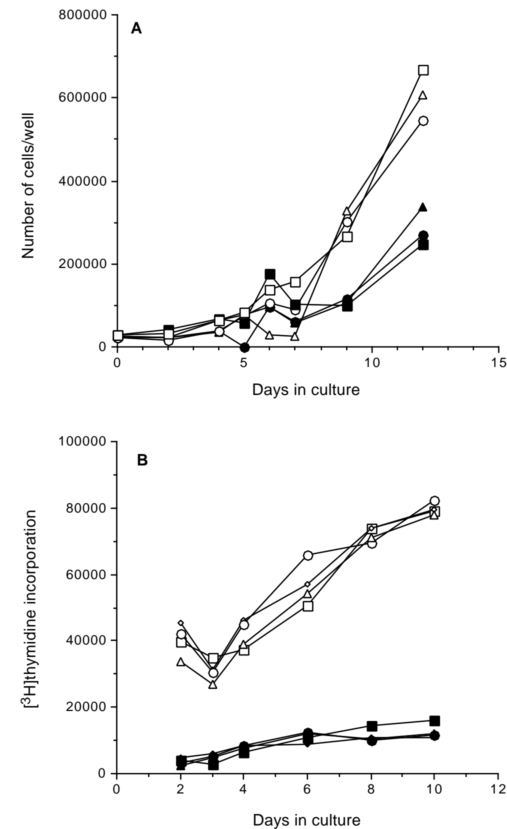

Fig. 10. Analysis of growth rates of control and antisense-expressing clones. (A) Growth rates of control, T-REP4 cells (squares) or antisense-expressing clones, 401 (triangles) or 706 (circles), were cultured for the indicated number of days on plastic (filled symbols) or plastic coated with collagen I (open symbols). Cells were quantitated by counting in triplicate. In each case, the standard deviation was less than 5%. Note that cells grew faster on collagen-coated surfaces than on plastic. There was no difference in the growth rates of cells with decreased $\alpha_2\beta_1$ integrin compared to control cells. (B) Growth rates of control, T-REP4 cells (squares) or antisense-expressing clones, 401 (triangles), 706 (circles), or 703 (small diamonds), that were cultured on a collagen-coated surface (open symbols) or in three-dimensional collagen gels (filled symbols). Cells were continuously labelled with [^{3}H]thymidine in order to quantitate cell growth. Note that culture in three-dimensional collagen gels dramatically slowed the growth rate of these cells relative to growth on planar collagen. There was no difference in the growth rates of cells with decreased $\alpha_2\beta_1$ integrin compared to control cells.

of an integrin on cell surfaces affects the interactions of cells with an ECM ligand. As expected, cells with the least $\alpha_2\beta_1$ integrin on their surface adhered least effectively to collagen substrata, while cells with more $\alpha_2\beta_1$ integrin on their surfaces adhered more effectively to collagen substrata. In contrast, motility on collagen did not directly correlate with the level of $\alpha_2\beta_1$ integrin on the cell surface, since only those cells whose adhesion to collagen and cell-surface $\alpha_2\beta_1$ integrin expression were of an intermediate level exhibited an increased motility on collagen. These results are consistent with those of Grzesiak et al. (1992), who found that calcium ions, which diminish $\alpha_2\beta_1$ integrin-mediated adhesion of fibroblasts to collagen, caused increased cell motility on collagen. A similar, optimal intermediate density of cell-surface $\alpha_5\beta_1$ integrin can be inferred from two different studies in CHO cells. CHO variants that express less than wild-type levels of $\alpha_5\beta_1$ integrin were less motile on fibronectin than wild-type cells (Bauer et al., 1992), while CHO cells overexpressing $\alpha_5\beta_1$ integrin were also less motile on fibronectin than wild-type cells (Giancotti and Ruoslahti, 1990). The optimal intermediate density of integrin on the surface of a motile cell may reflect a balance between the cell forming an interaction with collagen that is strong

enough to generate traction, and not so strong as to tightly tether the cell. A similar model was proposed to explain the role of certain cell-surface proteoglycans in cell motility (Culp et al., 1978; Ruoslahti, 1988). A cell-surface chondroitin sulfate proteoglycan enhances the motility of melanoma cells across collagen (Faassen et al., 1992), presumably by weakening adhesive contacts to collagen (Brennan et al., 1983; Yamagata et al., 1989; Faassen et al., 1992).

Cell-surface receptor density also plays a role in the morphological organization of mammary cells cultured in collagen gels. Duct-like structures formed by T47D cells in collagen gels were not formed by cells expressing decreased $\alpha_2\beta_1$ integrin levels. This suggests that a critical density of $\alpha_2\beta_1$ integrin receptors on the cell surface is required before a signal, not unidentified, is generated that results in morphological organization. Our results point to a crucial role for the $\alpha_2\beta_1$ integrin in mediating the in vitro morphogenesis of mammary cells. Since the $\alpha_2\beta_1$ integrin is expressed at high levels in mammary glands in vivo (Zutter et al., 1990), it is likely to be an important mediator of in vivo mammary gland morphogenesis. Using an antisense approach similar to that presented here, we found that collagen-mediated morphogenesis of another epithelial cell line, MDCK, is also dependent on expression of the $\alpha_2\beta_1$ integrin (E. Saelman et al., unpublished data). Additionally, the addition of α_2 integrin to mouse breast carcinoma cells that did not express the α_2 integrin resulted in decreased motility and tumorgenicity, and conferred on the cells the ability to organize in collagen gels (M. Zutter et al., unpublished data).

Our results with decreased $\alpha_2\beta_1$ integrin levels in breast carcinoma cells are in contrast to those where $\alpha_2\beta_1$ integrin was expressed on rhabdomyosarcoma (RD) cells (Chan et al., 1991). The expression of $\alpha_2\beta_1$ integrin by RD cells rendered them more metastatic. Since RD cells start out expressing no $\alpha_2\beta_1$ integrin, the experimentally transfected cells may acquire an optimal density of $\alpha_2\beta_1$ integrin and become motile. Alternatively, since skeletal muscle cells do not normally express $\alpha_2\beta_1$ integrin, $\alpha_2\beta_1$ integrin expression on RD cells represents aberrant expression that may have a functionally different outcome than on mammary cells.

Culture in three-dimensional collagen gels reduced the rate of cell division for T47D cells relative to that observed on planar collagen surfaces. The growth-regulatory properties of the ECM have been documented for a variety of cell types (Dike and Farmer, 1988; Woodley et al., 1990). This growth regulation was not altered for cells expressing decreased $\alpha_2\beta_1$ integrin levels. The residual level of $\alpha_2\beta_1$ integrin present on the antisense-expressing clones may be sufficient to generate the signals that regulate growth. If this is the case, then the critical density of $\alpha_2\beta_1$ integrin on the cell surface that is necessary to regulate cell growth is less than the density required to generate morphological organization in collagen gels. Alternatively, a receptor other than the $\alpha_2\beta_1$ integrin may be responsible for regulating growth in collagen gels. Indeed, when anti-α_2-integrin antibodies were used to block the morphological organization of T47D cells in collagen gels, there was no apparent increase in cell number compared with control cultures. This suggests that the growth-regulatory effect is not directly mediated through $\alpha_2\beta_1$ integrin. In contrast to our results, changes in α_5 integrin expression on CHO cells did affect growth rate (Giancotti and Ruoslahti, 1990), suggesting that the $\alpha_2\beta_1$ integrin and the $\alpha_5\beta_1$ integrin have different functions with regard to growth regulation.

Since normal breast epithelium is surrounded by a basement membrane, and we found no evidence that T47D cells secreted basement membrane components when cultured in collagen gels, the morphological organization that we note in these cultures is probably due to a direct interaction with collagen I. The $\alpha_2\beta_1$ integrin can bind to stromal collagen as well as to both of the basement membrane components, collagen IV and laminin. We found that gels composed of collagen I were much better at promoting tubule formation than were gels composed of Matrigel. Consistent with this observation, Petersen et al. (1992) found that T47D cells cultured in Matrigel did not form the spherical structures that are characteristic of primary mouse mammary cells cultured in Matrigel. Stromal collagen gels also induce primary mouse mammary morphogenesis, although Matrigel is better at inducing milk protein expression (Parry et al., 1985; Li et al., 1987). These differences may be due to the multiple growth factors present in Matrigel. Thus, studies using simpler gels composed of collagen I make it easier to determine the role of an $\alpha_2\beta_1$ integrin ligand on morphogenesis. The response of T47D cells to stromal collagen may be physiologically relevant, since the T47D cells were derived from a well-differentiated adenocarcinoma, and a hallmark of such tumors is the ability to form glandlike structures despite having invaded beyond the basement membrane. Additonally, there is a very high concentration of stromal collagen surrounding the mammary epithelium in the developing mouse mammary gland (Keely et al., unpublished data), suggesting that collagen plays an important role in mammary morphogenesis in vivo.

Our results are the first to successfully demonstrate decreased expression of a specific integrin alpha subunit using a stable antisense RNA technique. Other studies that affect the expression of integrins have also suggested a role for integrins in mediating cellular phenotype. Loss of β_1 integrin expression from F9 embryonal carcinoma cells by gene targeting severely disrupted morphological differentiation (Stephens et al., 1993). Additionally, antisense expression of the β_1 integrin resulted in a partial decrease in both the adhesion and growth of quail cells (Hayashi et al., 1991). The effects of decreasing the β_1 integrin subunit are difficult to interpret, however, since the β_1 integrin family includes receptors for most of the known ECM ligands. Decreases in both alpha and beta integrin subunits were obtained using antisense oligonucleotides to inhibit neural crest cell adhesion (Lallier and Bronner-Fraser, 1993). As the effect was temporary, they were never able to establish homogeneous cellular phenotypes with regard to receptor expression. Antisense approaches have also been successfully used to decrease E-cadherin expression (Vleminckx et al., 1991), thrombospondin levels (Castle et al., 1991) and the 32 kDa laminin receptor (Mafune and Ravikumar, 1992). The paucity of studies that have successfully used antisense techniques attests to the difficulty of this approach.

One advantage of generating clones with decreased α_2 integrin expression is that, unlike experiments with blocking antibodies, we were able to mimic the partial decrease in integrin expression noted in poorly differentiated mammary carcinomas. While function-blocking anti-human α_2 integrin antibodies exist, their use is limited in long term or in vivo investigations. Additionally, reports of cell-signalling events

that are triggered by antibodies (Menko and Boettiger, 1987; Werb et al., 1989; Zhou and Brown, 1994) suggest that interpretation of experiments using anti-integrin antibodies on living cells may not always be straightforward. A recent report of an inhibitory effect on one integrin by an antibody against a different integrin further attests to the complexity of these kind of studies (Blystone et al., 1994). Considering that such difficulties in interpreting antibody inhibition data may occur, genetic manipulation of cell-surface receptors is an important additional means to assess the functional roles of adhesion receptors.

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