Journal of Cell Science 107, 3557-3568 (1994)
Printed in Great Britain © The Company of Biologists Limited 1994

Branching morphogenesis of human mammary epithelial cells in collagen gels

Feodor Berdichevsky*, Deborah Alford, Brendan D'Souza and Joyce Taylor-Papadimitriou†

Imperial Cancer Research Fund, PO Box 123, 44 Lincoln's Inn Fields, London WC2A 3PX, UK

*Present address: Dana-Farber Cancer Institute, 44 Binney Street, Boston, MA 02115, USA
†Author for correspondence

SUMMARY

To study the morphogenesis of human epithelial cells in vitro we have used a three-dimensional collagen matrix and a newly developed mammary epithelial cell line, 1-7 HB2. In standard medium 1-7 HB2 cells formed compact balls/spheres inside collagen type I gels, while cocultivation with various fibroblast cell lines or growth in fibroblast-conditioned media resulted in the appearance of branching structures. At least two different soluble factors secreted by fibroblasts were found to be implicated in the branching morphogenesis. Firstly, hepatocyte growth factor/scatter factor could induce branching in a concentration-dependent manner. Moreover, a polyclonal serum against hepatocyte growth factor/scatter factor completely inhibited the branching morphogenesis induced by medium conditioned by MRC-5 fibroblast cells. In contrast, a morphogenetic activity secreted by human foreskin fibroblasts was identified that appears to be different from hepatocyte growth factor/scatter factor and from a number of other well-characterized growth factors or cytokines. This model system has been used to examine the role of integrins in mammary morphogenesis. The expression of the $\alpha_2\beta_1$, $\alpha_3\beta_1$ and $\alpha_6\beta_4$ integrins was decreased when cells were plated on collagen gels. The addition of specific blocking monoclonal antibodies directed to the α_2- and β_1-integrin subunits to growth media impaired cell-cell interactions and interfered with the formation of compact structures inside collagen gels, suggesting that the $\alpha_2\beta_1$ integrin can control intercellular adhesion in mammary morphogenesis. In contrast one of the blocking monoclonal antibodies against the α_3-integrin subunit (P1B5) mimicked the effect of soluble 'morphogens'. Our results suggest that the modulation of $\alpha_3\beta_1$ activity may represent an important event in the induction of branching morphogenesis of human mammary epithelial cells.

Key words: morphogenesis, mammary, collagen, epithelial, human

INTRODUCTION

Branching morphogenesis is an important stage in the development of different epithelial organs and the adjacent mesenchyme has been implicated in this process in the salivary gland (Nogawa and Mizuno, 1981), the kidney (Saxen and Sariola, 1987), and the mammary gland (Sakakura, 1987). Moreover, the inductive effect of mesenchymal tissue on morphogenesis can be reproduced in vitro when epithelial cells are cocultivated with fibroblasts (Montesano et al., 1991a,b). Although in most cases, exact mechanisms of the transmission of signals remain unknown, recent reports have suggested that soluble factors secreted by fibroblasts, interaction of epithelial cells with the fibroblast-deposited extracellular matrix (ECM) and a direct contact between fibroblasts and epithelial cells can contribute to this process (Montesano et al., 1983; Kratochwil et al., 1986; Nakanishi and Ishii, 1989; Montesano et al., 1991a,b; Hirai et al., 1992).

The mammary gland provides a particularly interesting model system for studies in morphogenesis since unlike other glandular organs, most of the development occurs post-natally. Thus in the rodent the effect of exogenously applied hormones and growth factors on the different stages of organogenesis can be studied even in vivo (Daniel et al., 1987, 1989; Shedeker et al., 1991). In addition, mammary organoids and mammary epithelial cells can be relatively easily isolated, and in vitro culture systems have been developed for maintenance and induction of morphological and functional differentiation (Emerman and Pitelka, 1977; Yang et al., 1979, 1980; Guzman et al., 1982; Richards et al., 1982; Lee et al., 1984; Barcellos-Hoff et al., 1989; Darcy et al., 1991). However, these in vitro studies have focused more on functional differentiation of rodent cells and less on morphogenesis of human cells and on defining molecules in the cell or in the environment that are involved in this process.

Integrins, which control interactions of cells with the ECM and with each other (Hynes, 1992) appear to play an important role in the development and differentiation of epithelial organs as well as in angiogenesis and myogenesis (Rosen et al., 1992; Sorokin et al., 1990; Roman et al., 1991; Kurpakus et al., 1991; Gamble et al., 1993). The fact that integrin expression in vitro can be regulated by some growth factors and cytokines

(Heino et al., 1989; Santala and Heino, 1991), suggests that integrin function may be one of the targets for the signals generated by fibroblast-produced morphogens in vivo. In previous studies we have shown that a cell line MTSV1-7 developed from epithelial cells cultured from human milk (Bartek et al., 1991) grow and form compact round structures when cultured in collagen type I gels (Berdichevsky and Taylor-Papadimitriou, 1991). MTSV1-7 cells also form ridges and structures from a monolayer around collagen fibres added in the medium and in this system morphogenesis was found to be dependent on the function of the $\alpha_2\beta_1$ integrin (Berdichevsky et al., 1992). We have now developed a new subline of the MTSV1-7 cells, 1-7 HB2, which provides a unique model system for analysing branching morphogenesis of human mammary epithelial cells in vitro. Here we describe this cell line and demonstrate that hepatocyte growth factor/scatter factor (HGF/SF) and another diffusible factor(s) secreted by fibroblasts induce branching of 1-7 HB2 cells inside collagen gels. Moreover, the results of an analysis of integrin expression in this system and of the effect of blocking antibodies to the integrin subunits suggests a role for the $\alpha_2\beta_1$ and $\alpha_3\beta_1$ integrins in the morphogenetic process.

MATERIALS AND METHODS

Antibodies and probes

The mouse monoclonal antibodies (mAbs) to α_1 (TS2/7) and α_4 (B-5G10) integrin subunits were provided by Dr M. E. Hemler (Dana-Farber Cancer Institute, Boston, MA), the mouse mAb to α_2 integrin subunit (HAS4) was from Dr F. Watt (Imperial Cancer Research Fund, London), the mouse mAb directed to α_2 integrin subunit (5E8) was kindly provided by Dr Bankert (Roswell Park Cancer Institute, Buffalo, NY), the rat mAbs to α_5 (mAb16) and β_1 (mAb13) integrin subunits were from Dr K. Yamada (NIH, Bethesda, MD), the rat mAbs to the α_6 integrin subunit (GOH3) were from Dr A. Sonnenberg (University of Amsterdam, Amsterdam), the mouse mAbs to the α_3 integrin subunit (J143) were from Dr A. Albino (Sloan-Kettering Institute, New York, NY), the mouse mAbs to the α_3 (IA3) integrin subunit will be characterized elsewhere (M. Shearer and F. Berdichevsky, personal communication). The mouse mAbs to the α_2 (P1E6), α_3 (P1B5) and β_4 (3E1) integrin subunits were purchased from Chemicon International Inc. (London, UK), and from Telios Pharmaceutical Inc (San Diego, CA). Polyclonal rabbit antibodies to recombinant human HGF (rhHGF) were kindly provided by the Mitsubishi Kasei Corporation (Yokohama, Japan). cDNA probes specific for α_2 (clone 2.72L) and α_3 (clone 3.10) integrin subunits were from Dr M. Hemler, the cDNA probe specific for the α_6 integrin subunit was from Dr A. Sonnenberg, and the cDNA probe specific for the β_1 integrin subunit was provided by Dr R. Hynes (Mass. Inst. Technology, Cambridge, MA). The additional cDNA probes specific for the α_2 integrin subunit were synthesised by RT-PCR on poly(A)$^+$ RNA prepared from MTSV 1-7 cells. Two pairs of oligonucleotide primers (5′-CGCGGATCCAAACCCAGCGCAACTACGGTC-3′ (sense), 5′-AAATTGCAGCCACAGAGTAACC-3′ (anti-sense) and 5′-CAG-CATTGAAGGTACTG-3′ (sense), 5′-TATCTGAGCATTCTTGTT-GACC-3′ (anti-sense)) were designed based on published sequence (Takada and Hemler, 1989). The PCR was performed in two stages: (a) one cycle of 5 minutes at 94°C, 2 minutes at 58°C and 5 minutes at 72°C; (b) 25 cycles of 1 minute at 94°C, 2 minutes at 58°C and 2 minutes at 72°C. The fragments, 1380 bp and 939 bp, were purified from low-melting point agarose, cut with *Bam*HI and *Pst*I, and with *Pst*I and *Hin*dIII, respectively, subcloned into Bluescript-pKS(+) and sequenced using the Sequenase version of the T7 DNA polymerase. These fragments were further joined in Bluescript-pKS(+) and the resulting *Xba*I-*Hin*dIII fragment (2038 bp) representing the 5′ end of the α_2 cDNA was used in some hybridization experiments in combination with the 2.72L probe.

Indirect immunofluorescence

Cells and frozen sections were fixed for 30 seconds in acetone that had been precooled to −20°C, and air-dried for 5 minutes. Subsequent incubations with specific mouse mAbs and FITC-conjugated secondary goat anti-mouse polyclonal Abs (DAKO Ltd, Bucks, UK) were followed by three washes with PBS. Sections were mounted and viewed on a Zeiss Axioscop microscope.

Cell culture

Human foreskin fibroblasts (HFF), human breast fibroblasts, murine Balb/c 3T3, Swiss 3T3, Swiss 3T3-LI fibroblasts, human mammary carcinoma cell lines 5.3.1.E and MDA-MB-231 were cultured in DMEM supplemented with 10% FCS (DMEM 10% FCS). MTSV 1-7, a human mammary epithelial cell line (Bartek et al., 1991) and its derivatives, 1-7 HB1 and 1-7 HB2, were routinely cultured in DMEM 10% FCS supplemented with hydrocortisone (5 μg/ml) and insulin (10 μg/ml) (referred to as standard medium or E4 in Figs 7, 8). A procedure for embedding and growth of epithelial cells in collagen type I has been described elsewhere (Berdichevsky and Taylor-Papadimitriou, 1991). In a typical cocultivation experiment 5×10^4-10^5 fibroblasts were plated on 35 mm tissue culture dishes initially in DMEM 10% FCS; after 3-4 hours, when the cells had attached and spread, growth medium was replaced with neutralized 0.25% collagen type I premixed with epithelial cells (5×10^2-10^3 cells/ml). After gelation the cells within the gel were further cultured either in standard medium, in serum-free fibroblast-conditioned medium (FCM), or in standard medium supplemented with a specified concentration of growth factor. Cells in gels were refed with fresh media every 4-5 days.

For experiments in which antibodies to rhHGF were used in collagen gel culture, the conditioned medium or standard medium supplemented with the purified rhHGF was incubated with the Ab to rhHGF, or a control Ab, HMFG-1, for 4 hours at 4°C before being used to feed cells that had been embedded in collagen gels. For the experiments with mAbs to integrins, cells were preincubated with the appropriate Abs for 15 minutes at room temperature before mixing with neutralized collagen solution and the antibody was present throughout the incubation. After embedding and addition of medium, the concentration of the antibody was diluted one in ten.

Preparation of conditioned medium

Medium conditioned by various cell lines and strains (MRC-5, Swiss 3T3, Swiss 3T3-LI, 5.3.1.E, MDA-MB-231, HFF, human breast fibroblasts) was used as a supplement in some experiments. The cells were first grown to confluency in DMEM-10% FCS then washed twice with serum-free DMEM and cultured for 72 hours in serum-free DMEM. The medium was then harvested, filtered through 0.2 μm filter, stored at 4°C and when used was supplemented with FCS, insulin and hydrocortisone to the appropriate concentration. Unless otherwise stated the conditioned medium was used undiluted.

Growth factors and hormones

rhHGF was a gift from the Mitsubishi Kasei Corporation (Yokohama, Japan). Keratinocyte growth factor (KGF) was from Promega (Southampton, UK) acidic fibroblast growth factor was from AMS Biotechnology (Oxfordshire, UK), basic fibroblast growth factor and platelet-derived growth factor were purchased from Amersham UK.

Transforming growth factor β1 and transforming growth factor α were from Calbiochem (Nottingham, UK). Epidermal growth factor was purchased from Sigma (Dorset, UK).

Cell surface ^{125}I labelling, immunoprecipitation and electrophoresis

For the primary characterisation of integrin expression by 1-7 HB2, cells were removed from plastic with EDTA (2 mM), washed twice with PBS (pH 7.4) and labelled with Na^{125}I in suspension using lactoperoxidase and glucose oxidase (Hubbard and Cohn, 1972). In the experiments when integrin expression was analysed in cells cultured on collagen gels, cells were removed from the gels (and from plastic in parallel) using 0.1% collagenase. The cells were syringed three times through a 25 gauge needle, washed in PBS, and labelled as described above. Labelled cell-surface proteins were extracted with lysis buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 5 mM $MgCl_2$, 1% NP-40, 2 mM PMSF, 20 mM leupeptin) and precipitated as previously described (Adams and Watt, 1990). Immunoprecipitated proteins were separated in a 6.5%-polyacrylamide gel (Laemmli, 1970) and visualized after exposure to Fuji X-ray films.

Cell-substratum adhesion experiments

The 1-7 HB2 cell-deposited matrix in 96-well plates for adhesion experiments was prepared according to the published protocol (Carter et al., 1991). Two variants of the collagen matrix were used for the experiments: (i) 96-well plates were coated overnight at 4°C with a solution of collagen type I (10 μg/ml); or (ii) a neutralized solution of collagen type I (2 mg/ml) was aliquoted into 48-well plates (0.2 ml/well) and left for 2 hours at 37°C to polymerize. The control and matrix-coated wells were blocked with 0.1% of heat-denatured BSA and washed twice with PBS prior to the addition of cells to the wells. 1-7 HB2 cells were detached with a solution of 2 mM EDTA and loaded with the fluorescent dye 2′,7′-bis(2-carbodyethyl)-5(6)-carbodyfluorescein (Molecular Probe Inc., Eugene, OR) for 30 minutes, washed twice with PBS and resuspended in DMEM. After preincubation with the appropriate mAb for 30 minutes at 4°C, cells were added to the matrix-coated dishes and left for 25-30 minutes at 37°C before washing three times with DMEM. The fluorescence before and after washes was measured with a CytoFluor 2300 fluorescence analyser (Millipore Co., Bedford, MA) and adhesion was calculated as the number of cells attached per mm^2 of coated surface.

RNA extraction and northern blot analysis

Total RNA from cells grown on plastic or on collagen gels was extracted according to the procedure described by Chomczynski and Sacchi (1987). After electrophoretic separation in a 1.2% formaldehyde gel RNA was transferred to a Hybond N+ membrane. Hybridization with ^{32}P-labelled probes and washing of filters were performed as previously described (Church and Gilbert, 1984).

RESULTS

Isolation of the 1-7 HB2 cell line

In order to study the role of integrins in branching morphogenesis of human mammary epithelial cells, we first established an in vitro experimental model system. Branching growth of mammary organoids has been observed to occur in collagen gels (Guzman et al., 1982; Foster et al., 1983) but the mixture of cell phenotypes, each with a distinctive pattern of integrin expression makes this system too complex, at least for initial analysis. Montesano and colleagues (1991a,b), however, have shown that an epithelial cell line (MDCK) can be induced by fibroblast-produced factors to form branching structures in collagen from the cysts that develop in normal medium. We therefore attempted to develop a human mammary epithelial cell line, HB2, which undergoes branching morphogenesis under these conditions.

The MTSV1-7 cell line, originally developed from breast epithelial cells cultured from human milk (Bartek et al., 1991), forms compact ball-like three-dimensional structures when grown in collagen type I gels (Berdichevsky et al., 1992), and when cocultivated inside the gels with human foreskin fibroblasts (HFF), branching structures occasionally appeared (10-20% of total colony numbers). After subcloning, two variants of MTSV 1-7 (1-7 HB1 and 1-7 HB2) were isolated where up to 90% of the colonies were observed to form branching structures in collagen gels when cocultivated with HFF for 8 days (Fig. 1). In other experiments we demonstrated that primary human breast fibroblasts (Chang et al., 1982), human embryonic lung fibroblasts (MRC-5) and three stable mouse fibroblastic cell lines (Balb/c 3T3, Swiss 3T3 and Swiss 3T3-LI) could also induce branching morphogenesis of 1-7 HB1 and 1-7 HB2 cells (not shown). In contrast, two human breast carcinoma cell lines of fibroblastic type, 5.3.1.E (Shearer et al., 1992) and MDA-MB-231, were unable to do so. Immunofluorescent staining confirmed that both subclones of MTSV1-7 cells showed the typical phenotype of luminal epithelial cells exhibited by the original cells, regardless of the growth conditions: they expressed the simple epithelial keratins (8, 18 and 19) and the polymorphic epithelial mucin, whereas the expression of keratin 14, collagen type IV or laminin was either weak or undetectable (data not shown). As 1-7 HB2 cells responded more dramatically to the presence of fibroblasts than the 1-7 HB1 cells, the subsequent studies concentrated on the HB2 subclone of the MTSV1-7 cells.

Fibroblasts produce a soluble factor(s) that induces branching morphogenesis of 1-7 HB2 cells

Previous studies have shown that fibroblasts embedded in collagen gels can induce mechanical forces that lead to gel contraction (Grinell and Lamke, 1984). In order to distinguish between ‘mechanical’ and ‘humoral’ influence of fibroblasts on morphogenesis, 1-7 HB2 cells were grown in collagen gels in the presence of HFF-conditioned medium (HFF-CM). As illustrated in Fig. 1, 1-7 HB2 cells grown in these conditions formed branching colonies almost as efficiently as they did in the cocultivation experiments. A more detailed examination of the development of branching structures over time showed that after 10-20 hours in collagen most of the single cells developed characteristic cytoplasmic processes. After 48-72 hours, primitive ‘star-like’ structures appeared where 2-3 cells with processes remained in contact with each other (Fig. 2A). Further proliferation resulted in progressive elongation of branches (Fig. 2B,C) that was largely completed after 8-10 days. Immunofluorescent staining of cross-sections revealed that over the time of observation (up to 14 days) all cells within the structures retained their luminal phenotype although only a small number of colonies developed an internal lumen (not shown). Similar results were obtained with conditioned media from MRC-5, Swiss 3T3, Swiss 3T3-LI cells, or fibroblasts cultured from adult breast tissue, but not with conditioned medium from the two breast cancer cell lines 5.3.1.E and MDA-MB.231. Fig. 3A quantitates the morphogenetic response to conditioned medium from MRC-5, HFF and 3T3 cells.

Studies of branching morphogenesis in MDCK cells have shown that the morphogenetic activity of fibroblast-conditioned medium is associated with HGF/SF (Montesano et al.,

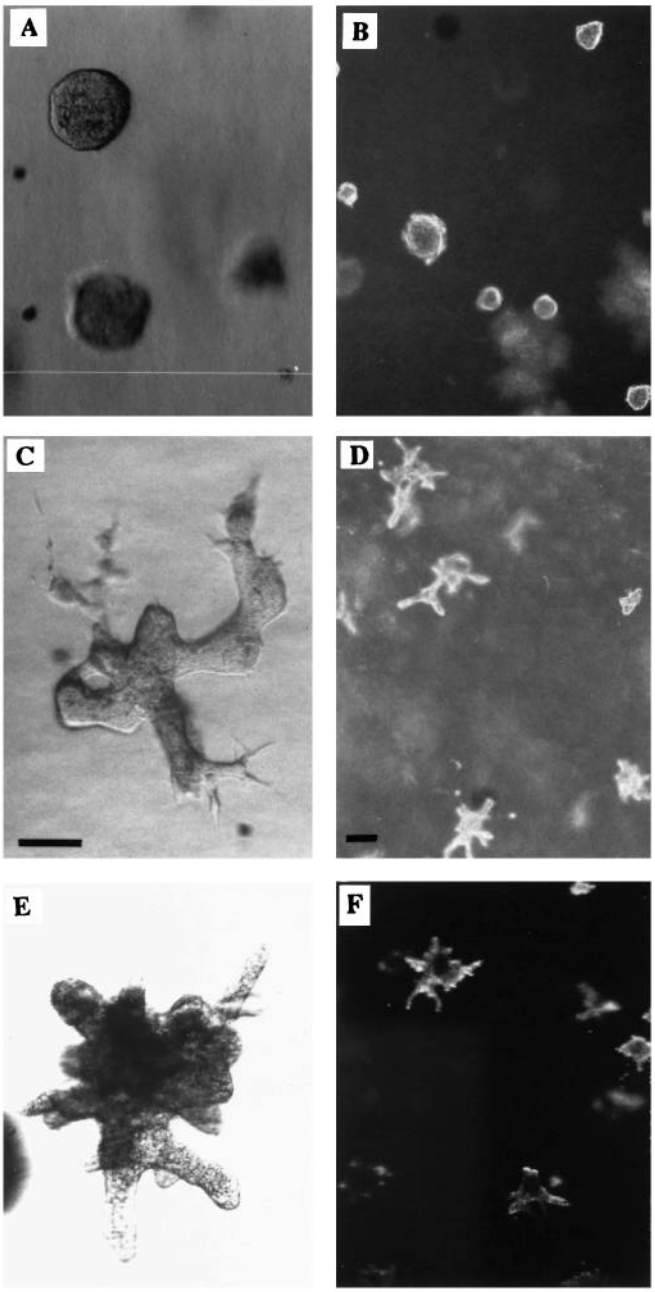

Fig. 1. Branching morphogenesis of 1-7 HB2 cells in collagen gels induced by co-culture with HFF or HFF-CM. 1-7 HB2 cells were embedded in collagen gels (see Materials and Methods) and cultured with unmodified standard medium, with HFF, or with medium conditioned by HFF for 8 days. Cells formed balls/spheres in unmodified medium (A,B) but formed branching colonies with HFF (C,D) or HFF-CM (E,F). Bar, 100 μm.

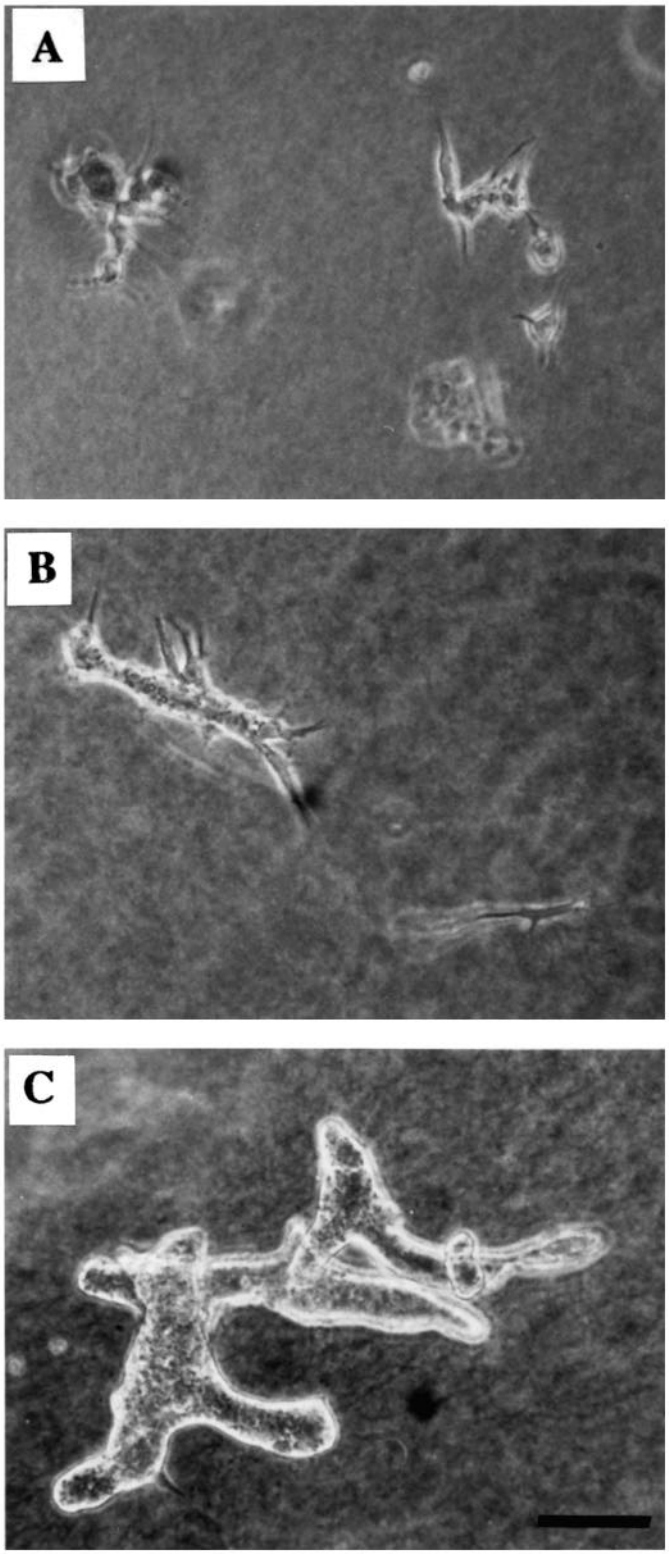

Fig. 2. Time course of development of branching structures from 1-7 HB2 cells grown in collagen gels with HFF-CM (A) 60 hours (B) 4 days, (C) 8 days after embedding. Bar, 100 μm.

1991b). Recombinant human HGF (rhHGF) added to normal medium was also found to induce branching morphogenesis of 1-7 HB2 cells in a concentration-dependent manner (Fig. 3A inset and Fig. 4A). To test whether the induction of branching of 1-7B2 cells by conditioned medium from different fibroblast cell lines is due to their secretion of HGF/SF, the effect of antibodies to rhHGF on the morphogenetic capacity of the conditioned medium was tested. When conditioned medium from MRC5 was supplemented with as little as 8 μg/ml of purified polyclonal antibodies against rhHGF the appearance of

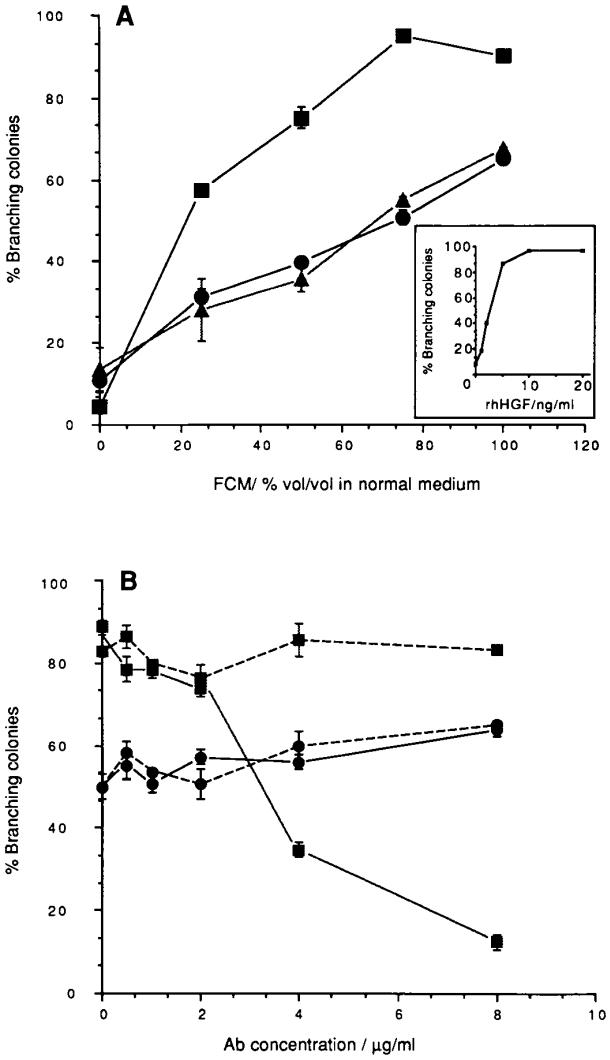

Fig. 3. Comparison of induction of branching morphogenesis by rhHGF and conditioned medium from HFF, MRC5 and 3T3 cells. (A) 1-7 HB2 cells were embedded in collagen gels and cultured with increasing amounts of conditioned medium from MRC5 (■), HFF (●) or Swiss 3T3 cells (▲). The proportion of colonies showing branching was estimated after 6 days. Insert shows dose-response for purified rhHGF. (B) Cells were cultured in collagen gels with conditioned medium from MRC5 (■) or HFF (●) with (—) or without (---) antibody directed to rhHGF. Medium was incubated with the Ab before adding it to cells embedded in collagen, and replenished at each media change. (See Materials and Methods). Proportion of colonies showing branching estimated at 6 days. A colony was recorded as a branching colony when 5 or more protuberances could be seen and at least one was as long as the body of the structure.

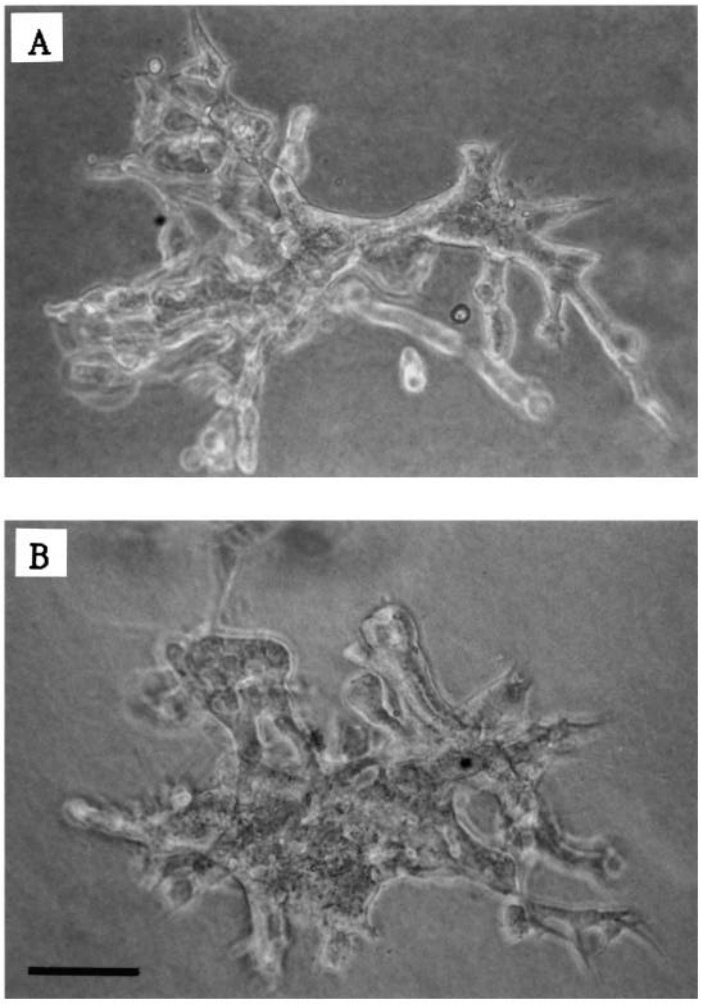

Fig. 4. Induction of branching morphogenesis by 1-7 HB2 cells cultured in collagen gels with (A) rhHGF or (B) antibody PIB5 to the α_3 integrin subunit. (A) Embedded cells were cultured in the presence of 10 ng/ml rhHGF. (B) Cells were pretreated for 15 minutes with the antibody P1B5 at a dilution of 1/100 before embedding in collagen gels and culturing with standard medium. The structures shown in the figure were photographed 7 days after embedding. Bar, 100 µm.

branching colonies was reduced to background level (Fig. 3B). However, in parallel experiments different concentrations of anti-rhHGF antibodies failed to block the branching induced by HFF-CM (Fig. 3B). Thus, it would appear that whereas the modulating effect of MRC5 fibroblasts on the morphogenesis was associated with the production of HGF/SF, the branching growth of 1-7 HB2 cells could also be induced by another factor(s) that is (are) secreted by HFF. Interestingly, the morphogenetic activity of conditioned medium from HFF was not abolished by heating media at 95°C for 3 minutes, a treatment that is known to inactivate HGF/SF. Moreover when added together in different concentrations, HGF and HFF-CM appeared to act synergistically. More and longer branches were observed for individual colonies, and maximum branching was observed with 2 ng/ml of HGF when 25% of the medium was HFF-CM. This also suggests that the active factor in HFF-CM is different from HGF.

The effect of several other growth factors on the morphogenesis of 1-7 HB2 cells in collagen gels was also analysed. Although EGF and TGFβ1 (at minimal concentrations 30 ng/ml and 20 ng/ml, respectively), were found to change the morphology of the colonies in collagen gels neither of these factors alone or the combinations shown in Table 1 were able to reproduce the effect of fibroblast-conditioned medium on morphogenesis of 1-7 HB2 cells. The growth rate of 1-7 HB2 cells in collagen was not stimulated by either HFF-CM or

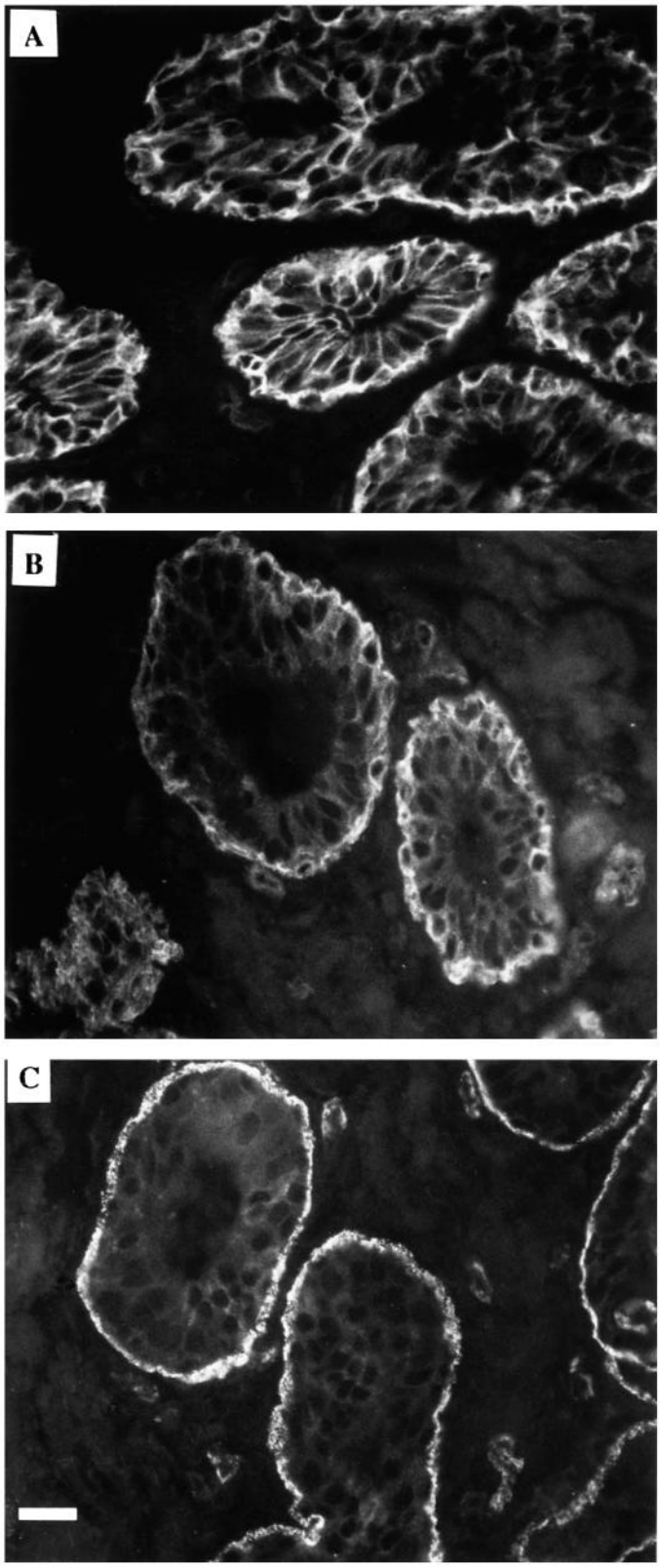

Fig. 5. Indirect immunofluorescent staining of tissue sections of resting normal breast with antibodies to the (A) α_2 (HAS4), (B) α_3 (J143), and (C) β_4 (3E1) integrin subunits. Bar, 40 μm.

Table 1. Factors tested that were unable to induce branching morphogenesis of 1-7HB2 cells

Factor*	Concentration (ng/ml)
Basic fibroblast growth factor (bFGF)	1-30
Acidic fibroblast growth factor (aFGF)	1-30
Platelet derived growth factor (PDGF)	1-20
Mouse hepatocyte growth factor (MHGF)	1-100
Transforming growth factor β (TGFβ)	0.1-20
Keratinocyte growth factor (KGF)	1-50
MHGF + TGFβ (10 ng/ml)	50
MHGF + KGF (20 ng/ml)	50
Epidermal growth factor (EGF)	10-100
EGF +bFGF (10 ng/ml)	50
KGF (+100 mg/ml of heparin)	1-50
KGF +EGF (10 ng/ml)	1-50
KGF +aFGF (10 ng/ml)	1-50

*Factors (at the concentrations indicated) were included in the medium added to the cells embedded in collagen gels.

HGF, although cell numbers were increased twofold in the presence of 30 ng/ml EGF (data not shown). This suggests that branching was not attributable to an effect on growth. It should be noted that mouse HGF did not induce branching, suggesting species specificity in the ligand-receptor interaction for the branching response, as has been noted for the induction of tumorigenicity by HGF (Rong et al., 1992).

Integrin expression in 1-7 HB2 cells and in mammary gland

Three members of the integrin family are found to be abundantly expressed in the resting and lactating mammary gland namely: $\alpha_2\beta_1$, $\alpha_3\beta_1$ and $\alpha_6\beta_4$ (Fig. 5 and Zutter and Santoro, 1990; Sonnenberg et al., 1990). Being a component of hemidesmosomes, the $\alpha_6\beta_4$ complex is found to be exclusively localized in the contacts of myoepithelial and luminal cells with the basement membrane (Fig. 5C). In contrast, the $\alpha_2\beta_1$ integrin is found evenly distributed along the basolateral cell surface in both cell layers (Fig. 5A). mAbs to the $\alpha_3\beta_1$ integrin showed strong staining of the membrane of basal cells (with a stronger staining in the cell-basement membrane contacts) and weaker staining of the basolateral surfaces of luminal cells. When analysed by immunoprecipitation (Fig. 6) or by flow cytometry and immunohistochemical staining (data not shown), the profile of integrin expression detected on the surface of 1-7 HB2 cells showed the $\alpha_2\beta_1$ and $\alpha_3\beta_1$ integrins to be expressed at high levels with the $\alpha_6\beta_4$ integrin also being detectable. This profile of integrin expression accurately reflects that seen in vivo and was the same when the cells were grown on plastic either in standard medium, in fibroblast-conditioned medium, or in medium supplemented with HGF/SF.

Regulation of integrin expression by collagen gels

To address the question of whether and how culture with collagen affects the integrin expression of 1-7 HB2 cells, the level of expression in cells grown on plastic was compared with the level in cells grown on collagen gels in standard medium, in HFF-CM and in medium supplemented with HGF/SF. Immunoprecipitation of cells that had been surface labelled with iodine after removal from the substrate showed that the cell surface level of all the integrins was decreased

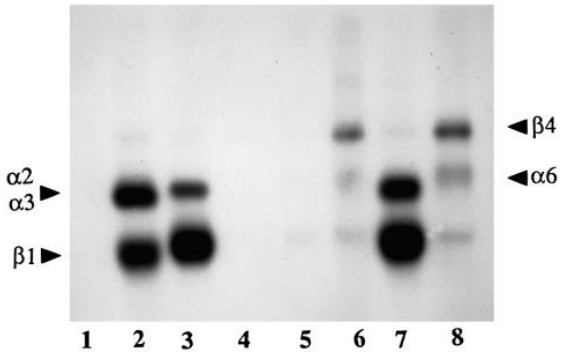

Fig. 6. Integrins expressed by 1-7 HB2 cells as detected by immunoprecipitation. Cells were surface labelled with ^{125}I using the lactoperoxidase method and integrins precipitated from samples of whole lysates containing equal numbers of counts using antibodies to integrin subunits. Integrins were eluted in Laemmli (1970) loading buffer and resolved in 7% gels in non-reducing conditions. Antibodies used: TS2/7-α_1 (lane 1); HAS4-α_2 (lane 2); J143-α_3 (lane 3); B5G10-α_4 (lane 4); mAb16-α_5 (lane 5); GOH3-α_6 (lane 6); MAR5-β_1 (lane 7); 3E1-β_4 (lane 8). NB, α_6-subunit usually runs between α_2, α_3 and β_1 bands.

in 1-7 HB2 cells grown on collagen gels (Figs 7, 8). For the $\alpha_2\beta_1$ and $\alpha_6\beta_4$ integrins the reduction in the expression was not affected by the growth media. However, the decrease in level of the $\alpha_3\beta_1$ integrin was more dramatic when cells were grown on collagen gels in HFF-CM (Fig. 7) or in medium with HGF/SF (Fig. 8). Northern blot analysis revealed that the specificity in the reduction of integrin expression of the α_3 integrin subunit in response to HGF/SF was also evident at the RNA level (Fig. 9). To analyse integrin function in the morphogenesis of 1-7 HB2 cells in more detail, the effect of specific blocking mAbs on the development of three-dimensional structures within collagen gels was examined.

Role of $\alpha_2\beta_1$ integrin in morphogenesis

At the concentrations shown in the legend to Fig 10, the dominant effect of treating 1-7 HB2 cells with blocking antibody to the α_2-(P1E6) or to β_1 (mAb13) subunits was to dissociate the structures formed in collagen gels whether cells were grown in standard medium or in fibroblast-conditioned media (Fig. 10C,D and G,H). Moreover, the addition of the blocking antibody to already preformed structures induced gradual weakening of cell-cell contacts and the appearance of loose colonies. In parallel experiments non-blocking anti-α_2 or anti-β_1 mAbs (HAS4 and MAR5, respectively) had no effect on morphogenesis. Thus despite the fact that the expression of $\alpha_2\beta_1$ integrin is down-regulated by the collagen gels, it appears that this integrin functions as an important regulator of cell-cell interactions in the morphogenesis of mammary epithelial cells. At higher concentrations of antibodies PIE6 or mAb13 to α_2 or β_1 integrin subunits, growth of HB2 cells in collagen gels was severely inhibited suggesting that some function of the $\alpha_2\beta_1$ integrin is necessary for growth in collagen. This function could relate to signals generated by cell-cell or cell matrix interactions or both.

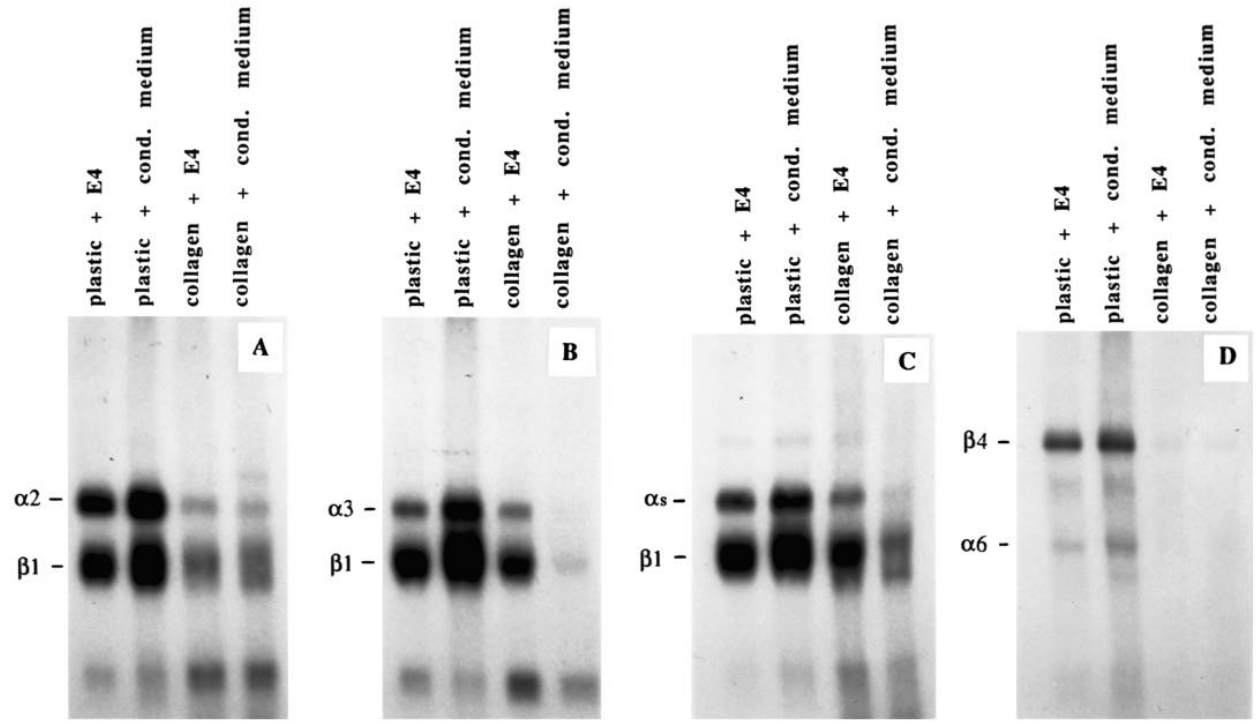

Fig. 7. Down-regulation of integrin expression in 1-7 HB2 cells cultured on collagen gels in standard medium (E4) and in the presence of HFF-CM. After culturing for 8 days under the conditions indicated, cells were removed from the plastic or collagen and surface labelled with ^{125}I. Samples of lysates containing equal numbers of counts were then precipitated with antibodies to the α_2 (HAS4) (A), α_3 (P1B5) (B), β_1 (MAR5) (C) and α_6 (GOH3) (D) integrin subunits and precipitates separated by 7% acrylamide gel electrophoresis in non-reducing conditions.

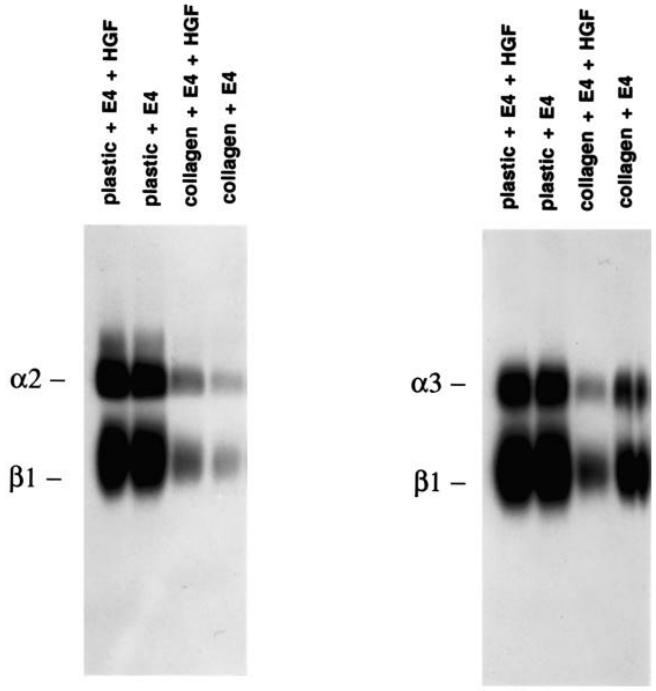

Fig. 8. Down-regulation of integrin expression in 1-7 HB2 cells cultured for 8 days on collagen in standard medium (E4) or in medium supplemented with 10 ng/ml of rhHGF. Experimental details as for Fig. 7.

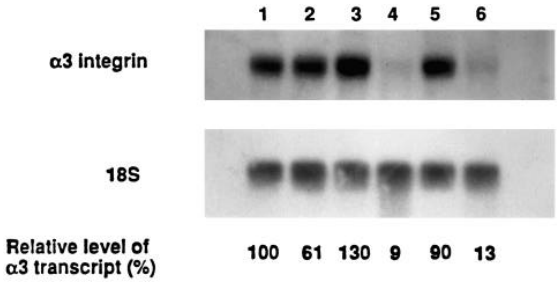

Fig. 9. Specific down-regulation of expression of the α_3 integrin subunit mRNA induced by HFF-CM or HGF in 1-7 HB2 cells grown on collagen gels. RNA was extracted from cells grown on collagen (lanes 2, 4 and 6) or on plastic (lanes 1, 3 and 5) for 24 hours in standard medium (lanes 1 and 2) or HFF-CM (lanes 3 and 4) or medium supplemented with 10 ng/ml rhHGF (lanes 5 and 6). Then 10 μg of RNA was run and blotted onto Hybond N+ membranes. The blot was probed sequentially with radioactive probes for the α_3 (clone 3.10) integrin mRNA and with a probe for 18 S RNA.

Antibody P1B5 to the α_3 integrin subunit induces branching morphogenesis of HB2 cells

The effect of two anti-α_3 mAbs (P1B5 and IA3) on morphogenesis was examined. Both P1B5 and IA3 inhibited the adhesion of 1-7 HB2 cells to a cell-deposited matrix, shown to be composed mainly of epiligrin (Carter et al., 1991), as detected by immunostaining with a specific antibody, BM165 (Marinkovich et al., 1992). However, only the P1B5 antibody inhibited cell attachment to polymerized collagen type I. (Fig. 11), and an effect on morphogenesis of 1-7 HB2 cells was seen only with the P1B5 antibody and not with the IA3 antibody. The striking effect of mAb P1B5 in standard medium was to induce branching in approximately 25% of the colonies, which resembled the branching induced by HGF/SF treatment (compare Fig. 4A,B). These data suggest that the down-regulation (or modulation) of the collagen-binding activity of the $\alpha_3\beta_1$ integrin may be required for branching morphogenesis inside collagen gels. It should be noted that the addition of the P1B5 antibodies alone did not have an inhibitory effect on the branching induced by HFF-CM (Fig. 10E,F) or HGF/SF (data not shown) although some colonies appeared to develop somewhat more elongated branches.

DISCUSSION

The three-dimensional collagen matrix has been previously used for studying morphological and functional differentiation of various epithelial tissues. A recent report by Montesano and colleagues (1991a) and our present data show that it can also be utilized to study a specific aspect of morphological differentiation, branching morphogenesis, under highly controlled conditions. This study specifically addresses two questions relating to morphological differentiation of the mammary gland: (i) the role of fibroblasts; and (ii) integrin function(s).

Soluble factor(s) secreted by fibroblasts induce branching morphogenesis of mammary epithelial cells

Although a role for surrounding mesenchyme in the process of branching morphogenesis in the developing mammary gland is well documented (Sakakura et al., 1976, 1982), the mechanisms underlying the effect have not been studied at the molecular level. In this report we show that fibroblasts, the major cellular component of mesenchyme, secrete morphogenetic factor(s) that can initiate branching morphogenesis of human mammary epithelial cells in collagen gels. These data strongly support the earlier hypothesis that modulating effects of mammary mesenchyme on morphogenesis can be transmitted by soluble morphogens.

One of the important conclusions that can be drawn from this study is that branching morphogenesis of mammary epithelial cells can be induced by two different fibroblast-produced soluble factors. HGF/SF was identified as the morphogenetic factor produced by MRC-5 cells, while some other factor (or factors) was (were) responsible for the morphogenetic activity in conditioned medium from HFF or 3T3 cells: HGF/SF and the HFF-produced factor(s) differ both antigenically and in physiochemical properties, and appear to act synergistically in stimulating branching of 1-7 HB2 cells in collagen gels.

HGF/SF is thought to be important in the development and remodelling of some epithelial organs (Sonnenberg et al., 1993; Zarnegar and Defrances, 1993), and although data describing the expression of HGF/SF in the mammary gland are not presently available, it was recently reported that the receptor for this cytokine, (the product of the met proto-oncogene) is found on the surface of ductal epithelial cells (Tsarfaty et al., 1992). Thus, it is possible that the HGF/SF-met interaction could govern certain stages of mammary morphogenesis in vivo.

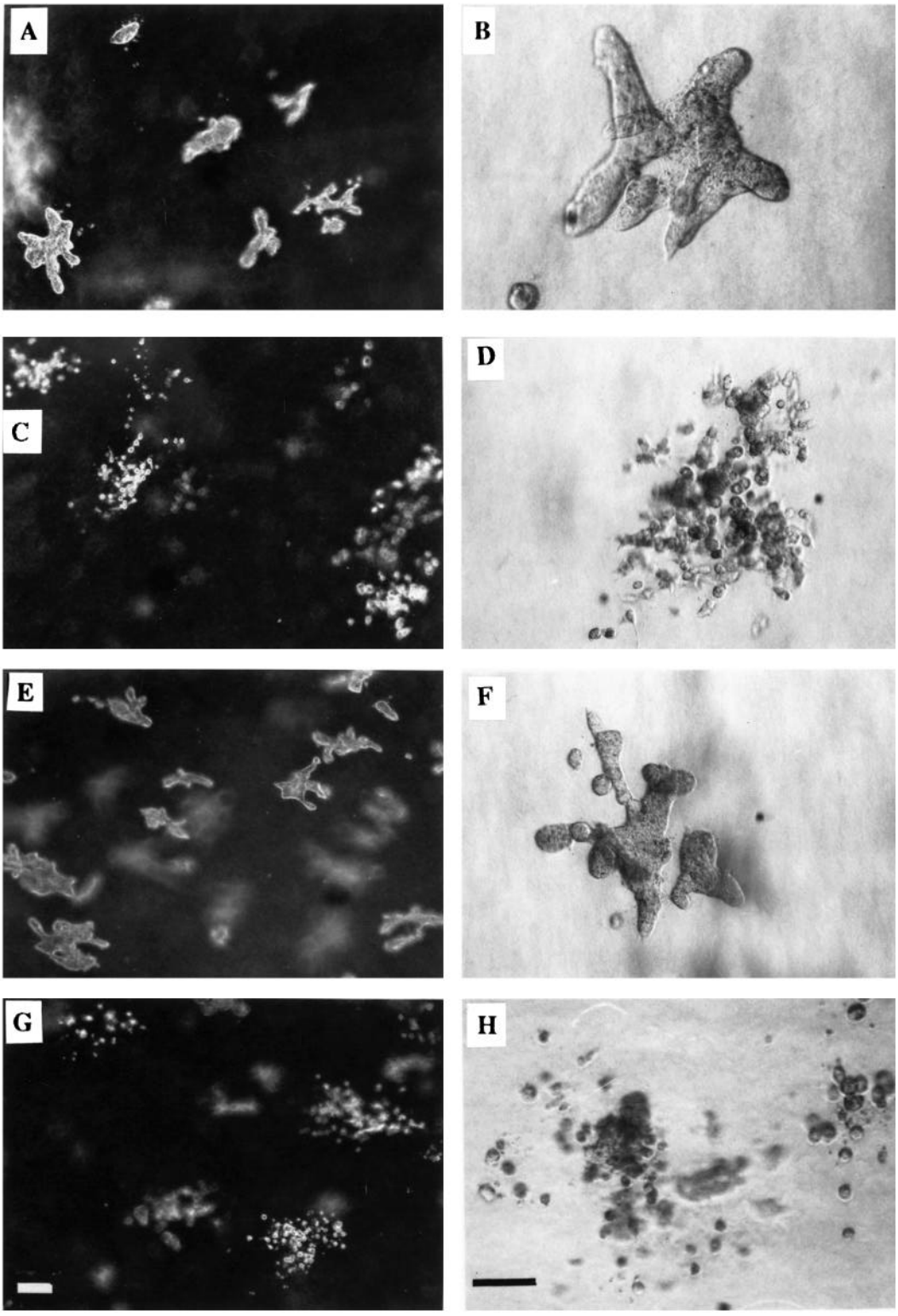

Fig. 10. Effect of antibodies to integrins on branching morphogenesis of 1-7 HB2 cells co-cultured in collagen gels with HFF-CM. Cells were pretreated with anti-integrin mAbs in HFF-CM (C-H) or left with no treatment (A,B) before embedding in collagen gels. The antibodies directed to α_2 (P1E6; C and D) and to β_1 (mAb13; G and H) subunits inhibited the formation of compact structures in collagen gels. The mAbs against α_3 (P1B5; E and F) subunit had no significant influence on branching morphogenesis. The antibody dilutions were as follows: 1:400 dilution of ascites for P1E6 and P1B5; 1 μg/ml for mAb13. Colonies were photographed 8 days after the embedding. Bar, 100 μm.

The question arises as to why the mammary gland may require the activity of two (or more) factors for branching morphogenesis. Unlike other organs, which exhibit branching morphogenesis only in the embryo or in the new born, branching of the structures in mammary gland also occurs in adult life at puberty and pregnancy. It is therefore conceivable that different morphogenetic factors produced by mesenchyme may be involved at different stages of development. Further characterization of the factor(s) produced by HFF is clearly warranted.

Integrins in morphogenesis of 1-7 HB2 cells in collagen gels

Our data represent the first attempt to understand the specific

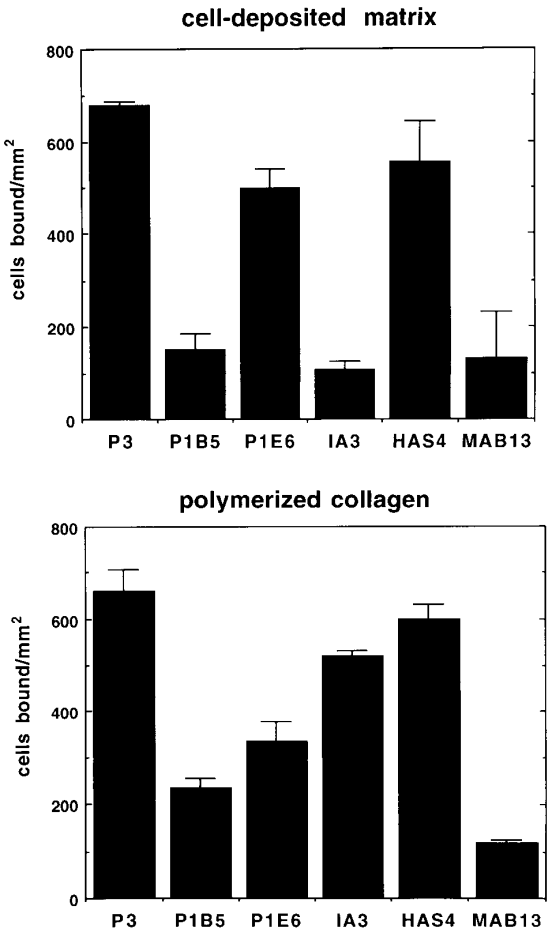

Fig. 11. Inhibition of adhesion of 1-7 HB2 cells to polymerized collagen and to a cell-deposited matrix by mAbs to integrins. Before plating on the matrix-coated surface 1-7 HB2 cells were preincubated either with the control lgG1 mAbP3 or with anti-α_2 (P1E6, HAS4,) anti-α_3 (P1B5, 1A3) and anti-β_1 (mAb13) mAbs. Cells were allowed to adhere for 25 minutes and non-attached cells were removed in 3 subsequent washes. The final concentration of mAbs were 1:200 dilution of ascites for P1B5 and P1E6, 1:500 dilution of ascites for mAb13, 20 μg/ml for HAS4 and IA3, 1:5 dilution of culture supernatant for P3.

role of integrins in branching morphogenesis. The 1-7 HB2 cells expressed the integrins found in mammary epithelial cells in vivo i.e. $\alpha_2\beta_1$, $\alpha_3\beta_1$, $\alpha_6\beta_4$. An important observation was that expression of all of these integrins was decreased when the cells were grown on collagen gels. Since both the $\alpha_2\beta_1$ and $\alpha_3\beta_1$ integrins were found to mediate adhesion of 1-7 HB2 cells to polymerised collagen, a weakening of these interactions might be expected to accompany branching when the cells become more motile inside the gels. A more detailed analysis using blocking antibodies, however, suggested specific roles for the $\alpha_2\beta_1$ and $\alpha_3\beta_1$ integrins as regulators of cell-cell and/or cell matrix interactions.

$\alpha_2\beta_1$ integrin

The presence of specific anti-α_2 (or anti-β_1) blocking antibodies in growth media prevented or disrupted the formation of compact three-dimensional structures in collagen gels implying that in spite of its down-regulation, the function of the $\alpha_2\beta_1$ integrin as a cell adhesion receptor was crucial in the morphogenesis. The localisation of the $\alpha_2\beta_1$ integrin on the lateral surface of epithelial cells in resting and lactating mammary gland (Fig. 5) also suggests that it can contribute to cell-cell interactions in vivo. Moreover, we find that in oncogenically transformed human mammary cells and in cell lines developed from the naturally occurring breast tumors, a decreased level of $\alpha_2\beta_1$ integrin expression correlated with the deterioration of intercellular contacts and the inability of the cells to form organised three-dimensional structures in collagen gels (Shearer et al., 1992; D'Souza et al., 1993).

In epithelial tissues homophilic cell adhesion is typically thought to be associated with the function of cadherins, a family of Ca^{2+}-dependent transmembrane molecules (Takeichi, 1990). However, when cadherin function was depleted in human keratinocytes by culturing in low Ca^{2+} medium, the integrins of the β_1 family (in particular, $\alpha_2\beta_1$ and $\alpha_3\beta_1$) were found to control cell-cell interactions of human keratinocytes (Larjava et al., 1990; Symington et al., 1993). Thus, our finding that $\alpha_2\beta_1$-integrin may contribute to the intercellular adhesion of epithelial cells, under the conditions of morphogenesis described here suggests that the function of cadherins might also be down-regulated. Preliminary experiments suggest that this is indeed the case (B. D'Souza, personal communication).

To demonstrate the effect of blocking antibodies to the $\alpha_2\beta_1$ integrin on cell-cell adhesion, the concentration of the antibodies was crucial. Higher concentrations than those shown in Fig. 10 resulted in inhibition of the growth of 1-7 HB2 cells in collagen, suggesting that some residual function of the integrin was required for growth under these conditions. This function could relate to signals generated by cell-cell or cell-matrix interactions or both. Whatever the cause, the growth inhibitory effect of antibodies to the $\alpha_2\beta_1$ integrin seen in collagen emphasises the crucial role of this integrin in the development of structure.

It should also be pointed out that the cell-dissociative effect of blocking antibodies to the $\alpha_2\beta_1$ integrin could be due to indirect effects. Our present data and earlier reports showed that collagen could modulate gene expression in mammary cells (Streuli and Bissel, 1990). Therefore, it is possible that in its interaction with collagen the $\alpha_2\beta_1$ integrin is generating signals that affect the functional activity of other cell adhesion molecules expressed by cells and that would also be negated by blocking anti-α_2 antibodies.

$\alpha_3\beta_1$ integrin

Our results also suggest that changes in the functional activity of the $\alpha_3\beta_1$ integrin may be involved in mammary morphogenesis. The blocking of binding of 1-7 HB2 cells to polymerized collagen by antibody P1B5 demonstrates that the $\alpha_3\beta_1$ integrin in these cells can bind to collagen. Accordingly, the down-regulation of $\alpha_3\beta_1$ expression in 1-7 HB2 cells grown on collagen gels in the presence of HGF/SF (or fibroblast-conditioned media) or inhibition of the $\alpha_3\beta_1$ collagen interaction with the P1B5 mAb could destabilise cell-collagen interaction and facilitate branching outgrowth. It is likely that branching morphogenesis in general requires a more dynamic interaction of cells with the ECM and with each other. The fact that the ECM and soluble morphogens produced by mesenchymal cells

can cooperatively modulate integrin functions in vitro strongly suggests that similar processes may be occurring in vivo and could be crucial for the branching development in various epithelial organs including the mammary gland.

We are grateful to the individuals who provided us with anti-integrin mAbs and cDNA probes and to the Mitsubishi Kasei corporation for supplying us with rhHGF and antiserum to hepatocyte growth factor. We also thank Dr Stefania Martignone for her help with some of the experiments using fibroblast-conditioned media and Dr Qilong Lu for examination of sections of structures formed by 1-7 HB2 cells grown in collagen gels. Feodor Berdichevsky is grateful to Dr M. Hemler for his support in the final stages of this work.

REFERENCES

Adams, J. C. and Watt, F. M. (1990). Changes in keratinocytes - extracellular matrix interactions during terminal differentiation: reduction in fibronectin binding precedes loss of the α5β1 integrin from the cell surface. *Cell* **63**, 425-435.

Barcellos-Hoff, M. H., Aggeler, J., Ramand, T.G. and Bissel, M. J. (1989). Functional differentiation and alveolar morphogenesis of primary mammary epithelial cells cultured on reconstituted basement membrane. *Development* **105**, 223-235.

Bartek, J., Bartkova, J., Kyprianou, N., Lalani, E. N., Staskova, Z., Shearer, M., Chang, S. and Taylor-Papadimitriou, J. (1991). Efficient immortalization of luminal epithelial cells from human mammary gland by introduction of simian virus 40 large tumor antigen with a recombinant retrovirus. *Proc. Nat. Acad. Sci USA* **88**, 3520-3524.

Berdichevsky, F. and Taylor-Papadimitriou, J. (1991). Morphological differentiation of hybrids of human mammary epithelial cell lines is dominant and correlates with the pattern of expression of intermediate filaments. *Exp. Cell Res.* **194**, 267-274.

Berdichevsky, F., Gilbert, C., Shearer, M. and Taylor-Papadimitriou, J. (1992). Collagen-induced rapid morphogenesis of human mammary epithelial cells: The role of $\alpha_2\beta_1$ integrin. *J. Cell Sci.* **102**, 437-446.

Carter, W., Ryan, M. and Gahr P. (1991). Epiligrin, a new cell adhesion ligand for integrin $\alpha_3\beta_1$ in epithelial basement membrane. *Cell* **65**, 599-610.

Chang, S., Keen, J., Lane, E. and Taylor-Papadimitriou, J. (1982). Establishment and characterization of SV-40 transformed human breast epithelial cell lines. *Cancer Res.* **42**, 2040-2053.

Chomczynski, P. and Sacchi, N. (1987). Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Anal. Biochem.* **162**, 156-159.

Church, G. and Gilbert, W. (1984). Genomic sequencing. *Proc. Nat. Acad. Sci. USA* **81**, 1991-1995.

Daniel, C., Silberstein, G. and Strickland, P. (1987). Direct action of 17 beta-estradiol on mouse mammary duct analyzed by sustained release implants and steroid autoradiography. *Cancer Res.* **47**, 6052-6057.

Daniel, C., Silberstein, G., Horn, K., Strickland, P. and Robinson, S. (1989). TGF-β1-induced inhibition of mouse mammary ductal growth: developmental specificity and characterization. *Dev. Biol.* **135**, 20-30.

Darcy, K. M., Black, J. D., Hahm, H. A. and Ip, M. M. (1991). Mammary organoids from immature virgin rats undergo ductal and alveolar morphogenesis when grown within a reconstituted basement membrane. *Exp. Cell Res.* **196**, 49-65.

D'Souza, B., Berdichevsky, F. B., Kyprianou, N. and Taylor-Papadimitriou, J. (1993). Collagen-induced morphogenesis and expression of the α_2-integrin subunit is inhibited in c-erbB2-transfected human mammary epithelial cells. *Oncogene* **8**, 1797-1808.

Emerman, J. T. and Pitelka, D. R. (1977). Maintenance and induction of morphological differentiation in dissociated mammary epithelium on floating collagen membranes. *In vitro* **13**, 316-328.

Foster, C., Smith, C., Dinsdale, E., Monaghan, P. and Neville, A. (1983). Human mammary gland morphogenesis in vitro: the growth and differentiation of normal breast epithelium in collagen gel cultures defined by electron microscopy monoclonal antibodies and autoradiography. *Dev. Biol.* **96**, 197-216.

Gamble, J. R., Matthias, L. J., Meyer, G., Kaur, P., Russ, G., Faull, R., Berndt, M. C. and Vadas, M. A. (1993). Regulation of in vitro capillary tube formation by anti-integrin antibodies. *J. Cell Biol.* **121**, 931-943.

Grinell, F. and Lamke. C. (1984). Reorganization of hydrated collagen lattices by human skin fibroblasts. *J. Cell Sci.* **66**, 51-63.

Guzman, R., Osborn, R., Yang, J., DeOme, K. and Nandi, S. (1982). Transplantation of mouse mammary epithelial cells grown in primary collagen gel culture. *Cancer Res.* **42**, 2376-2383.

Heino, J., Ignotz, R. A., Hemler, M. E., Crouse, C. and Massague, J. (1989). Regulation of cell adhesion receptors by transforming growth factor-β. Concomitant regulation of integrins that share a common β1 subunit. *J. Biol. Chem.* **264**, 380-388.

Hirai, Y., Takebe, K., Kabayashi, S. and Takeichi, M. (1992). Epimorphin: a mesenchymal protein essential for epithelial morphogenesis. *Cell* **69**, 471-481.

Hubbard, A. and Cohn, Z. (1972). Externally disposed plasma membrane proteins. I. Enzymatic iodination of mouse L cells. *J. Cell Biol.* **64**, 438-460.

Hynes, R. (1992). Integrins: versatility, modulation and signalling in cell adhesion. *Cell* **69**, 11-25.

Kratochwil, K., Dziadek, M., Lohler, J., Harbers, K. and Jaenisch, R. (1986). Normal epithelial branching morphogenesis in the absence of collagen I. *Dev. Biol.* **117**, 596-606.

Kurpakus, M. A., Quaranta, V. and Jones, J. C. A. (1991). Surface relocation of α6β4 integrins and assembly of hemidesmosomes in an in vitro model of wound healing. *J. Cell Biol.* **115**, 1737-1750.

Laemmli, U. K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**, 680-685.

Larjava, H., Peltonen, J., Akiyama, S. K., Yamada, S. S., Galnick, H. R., Uitto, J. and Yamada, K. M. (1990). Novel function for β_1 integrins in keratinocytes cell-cell interactions. *J. Cell Biol.* **110**, 803-815.

Lee, E. Y. H., Parry, G. and Bissel, M. J. (1984). Modulation of secreted proteins of mouse mammary epithelial cells by the extracellular matrix. *J. Cell Biol.* **98**, 146-155.

Marinkovitch, M. P., Lunstrum, G. P., Keene, D. R. and Burgeson, R. E. (1992). The dermal-epidermal junction of human skin contains a novel laminin variant. *J. Cell Biol.* **119**, 695-703.

Montesano, R., Mouron, P., Amherdt, M. and Orci, L. (1983). Collagen matrix promotes reorganization of pancreatic endocrine cell monolayer into islet-like organoids. *J. Cell Biol.* **97**, 935-939.

Montesano, R., Schaller, G. and Orci, L. (1991a). Induction of epithelial tubular morphogenesis in vitro by fibroblast-derived soluble factors. *Cell* **66**, 697-711.

Montesano, R., Matsumoto, K., Nakamura, T. and Orci, L. (1991b). Identification of a fibroblast-derived epithelial morphogen as hepatocyte growth factor. *Cell* **67**, 901-908.

Nakanishi, Y. and Ishii, T. (1989). Epithelial shape change in mouse embryonic submandibular gland: modulation by extracellular matrix components. *BioEssays* **11**, 163-167.

Nogawa, H. and Mizuno, T. (1981). Mesenchymal control over elongating and branching morphogenesis in salivary gland development. *J. Embryol. Exp. Morphol.* **66**, 209-221.

Richards, J., Guzman, R., Konrad, M., Yang, J. and Nandi, S. (1982). Growth of mouse mammary gland end buds cultured in a collagen gel matrix. *Exp. Cell Res.* **141**, 433-443.

Roman, J., Little, C. and McDonald, J. (1991). Potential role of RGD-binding integrins in mammalian lung branching morphogenesis. *Development* **112**, 551-558.

Rong, S., Bodescot, M., Blair, D., Dunn, J., Nakamura, T., Mizuno, K., Park, M., Chan, A., Aaronson, S. and Vande-Woude, G. F. (1992). Tumorigenicity of the met Proto-Oncogene and the gene for hepatocyte growth factor. *Mol. Cell Biol.* **12**, 5152-5158.

Rosen, G., Sanes, J., LaChance, R., Cunningham, J., Roman, J. and Dean, D. (1992). Roles for the integrin VLA4 and its counter receptor VCAM-1 in myogenesis. *Cell* **69**, 1107-1119.

Sakakura, T., Nishizuka, Y. and Dawe, C. (1976). Mesenchyme-dependent morphogenesis and epithelium-specific cytodifferentiation in mouse mammary gland. *Science* **194**, 1439-1441.

Sakakura, T., Sakagami, Y. and Nishizuka, Y. (1982). Dual origin of mesenchymal tissues participating in mouse mammary gland embryogenesis. *Dev. Biol.* **91**, 202-207.

Sakakura, T. (1987). Mammary embryogenesis. In *The Mammary Gland: Development, Regulation and Function* (ed. M. Melvill and C. Daniel), pp. 37-65. Plenum Press New York.

Santala, P. and Heino, J. (1991). Regulation of integrin type cell adhesion receptors by cytokines. *J. Biol. Chem.* **266**, 23505-23509.

Saxen, L. and Sariola, H. (1987). Early organogenesis of the kidney. *Pediatr. Nephrol.* **1**, 385-392.

Shearer, M., Bartkova, J., Bartek, J., Berdichevsky, F., Barnes, D., Millis, R. and Taylor-Papadimitriou, J. (1992). Studies of clonal cell lines developed from primary breast cancers indicate that the ability to undergo morphogenesis in vitro is lost early in malignancy. *Int. J. Cancer* **51**, 602-612.

Shedeker, S., Brown, C. and DiAugustine, R. (1991). Expression and functional properties of transforming growth factor alpha and epidermal growth factor during mouse mammary gland ductal morphogenesis. *Proc. Nat. Acad. Sci. USA* **88**, 276-280.

Sonnenberg, A., Linders, C. J. T., Daams, J. H. and Kennel, S. J. (1990). The $\alpha_6\beta_1$ (VLA6) and $\alpha_6\beta_4$ protein complexes: tissue distribution and biochemical properties. *J. Cell Sci.* **96**, 207-217.

Sonnenberg, E., Weidner, K. M. and Birchmeier, W. (1993). Expression of the met receptor and its ligand, HGF-SF during mouse embryogenesis. In *Hepatocyte Growth Factor - Scatter Factor (HGF-SF) and the c*-met *Receptor* (ed. I. D. Goldberg and E. M. Rosen), pp. 381-394. Birkhäuser Verlag Publication.

Sorokin, L., Sonnenberg, A., Aumailley, M., Timpl, R. and Ekblom, P. (1990). Recognition of the laminin E8 cell-binding site by an integrin possessing the α_6 subunit is essential for epithelial polarization in developing kidney tubules. *J. Cell Biol.* **111**, 1265-1273.

Streuli, C. H. and Bissel, M. J. (1990). Expression of extracellular matrix components is regulated by substratum. *J. Cell Biol.* **110**, 1405-1415.

Symington, B. E., Takada, Y. and Carter, W. G. (1993). Interaction of integrins $\alpha_2\beta_1$ and $\alpha_3\beta_1$: potential role in keratinocyte intercellular adhesion. *J. Cell Biol.* **120**, 523-535.

Takada, Y. and Hemler, M. E. (1989). The primary structure of the VLA-2/collagen receptor α_2 subunit: homology to other integrins and the presence of possible collagen binding domain. *J. Cell Biol.* **109**, 397-407.

Takeichi, M. (1990). Cadherins: a molecular family important in selective cell-cell adhesion. *Annu. Rev. Biochem.* **59**, 237-252.

Tsarfaty, I., Resau, J., Rulong, S., Keydar, I., Faletto, D. and Vande-Woude, G. (1992). The met protooncogene receptor and lumen formation. *Science* **257**, 1258-1261.

Yang, J., Richards, J., Bowman, P., Guzman, R., Enami, J., McCormick, K., Hamamoto, S., Pitelka, D. and Nandi, S. (1979). Sustained growth and three dimensional organisation of primary mammary tumour epithelial cells embedded in collagen gels. *Proc. Nat. Acad. Sci. USA* **76**, 3401-3405.

Yang, J., Guzman, R., Richards, J., Jentoft, V., DeVault, M., Wellings, S. and Nanadi, S. (1980). Primary culture of human mammary epithelial cells embedded in collagen gel. *J. Nat. Cancer Inst.* **65**, 337-343.

Zarnegar, R. and Defrances, M. C. (1993). Expression of HGF-SF in normal and malignant human tissues. *Exp. Suppl.* **65**, 181-199.

Zutter, M. M. and Santoro, S. A. (1990). Widespread histologic distribution of the $\alpha_2\beta_1$ integrin cell surface collagen receptor. *Am. J. Pathol.* **137**, 113-120.

(Received 17 May 1994 - Accepted 1 August 1994)