Loss of MDCK cell $\alpha 2\beta 1$ integrin expression results in reduced cyst formation, failure of hepatocyte growth factor/scatter factor-induced branching morphogenesis, and increased apoptosis

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SUMMARY

Cellular interactions with collagen in a model of kidney tubulogenesis were investigated using Madin-Darby canine kidney (MDCK) cells in an in vitro morphogenetic system. MDCK cells adhered to collagen types I and IV in a Mg$^{2+}$-dependent manner, typical of the $\alpha 2\beta 1$ integrin. Collagen-Sepharose affinity chromatography and immunoblotting demonstrated the presence and collagen binding activity of the $\alpha 2\beta 1$ integrin on MDCK cells. To assess the function of $\alpha 2\beta 1$ integrin, MDCK cells were transfected with a plasmid pRVSv$\alpha 2$ which allowed the expression of $\alpha 2$-integrin subunit antisense RNA. Three G418-resistant clones showing reduced adhesion to collagen, stable genomic integration of the antisense construct, decreased $\alpha 2$-integrin subunit mRNA and decreased $\alpha 2$-integrin subunit protein expression were selected for analysis in morphogenetic experiments. MDCK cells and plasmid-only control transfectants, cultured in three-dimensional collagen type I gels, showed normal cyst formation, whereas the antisense RNA transfectants showed increased apoptosis and formed small rudimentary cysts. Stimulation with hepatocyte growth factor/scatter factor-containing 3T3 fibroblast-conditioned medium or recombinant hepatocyte growth factor/scatter factor resulted in extensive branching of the preformed control cysts whereas the surviving small cysts formed by antisense expressing cells increased in size but failed to elongate and branch upon stimulation. We conclude that $\alpha 2\beta 1$ integrin collagen interactions play a crucial role in the hepatocyte growth factor/scatter factor-induced tubulogenesis and branching morphogenesis of MDCK cells in collagen gels as well as an important role in cell survival.

Key words: morphogenesis, integrin, antisense RNA, hepatocyte growth factor/scatter factor, apoptosis

INTRODUCTION

Organization of epithelial cells into higher order structures requires a complex series of morphogenetic events (Rodriquez-Boulan and Nelson, 1989; Gumbiner, 1992). Three possible modes of morphogenetic signal transmission have been postulated (Grobstein, 1956; Saxén et al., 1976): cell-extracellular matrix (ECM) interactions (Ekblom, 1981; Saxén, 1987; Laurie et al., 1989; Wang et al., 1990a,b), cell-cell interactions (Takeichi, 1991; Hirai et al., 1992) and diffusion of soluble factors (Grobstein, 1956; Saxén et al., 1976). In recent years, a fibroblast-derived soluble factor, identified as hepatocyte growth factor/scatter factor (HGF/SF), has been shown to induce tubulogenesis by Madin-Darby canine kidney (MDCK) epithelial cells (Montesano et al., 1991a,b) providing important molecular data on the role of diffusible factors in epithelial morphogenesis (Birchmeier and Birchmeier, 1993). Also, additional studies indicated that the MDCK morphogenetic model is relevant to kidney development. Coculturing MDCK cells, seeded in collagen type I, with the mouse embryonic kidney induced branching morphogenesis (Santos et al., 1994). Furthermore, antibodies directed against HGF/SF markedly inhibited embryonic kidney induced morphogenesis.

Growing evidence has indicated that, in addition to soluble cytokines, ECM components such as collagen and laminin mediate, at least in part, the organization of epithelial cells into three-dimensional structures (Bennett, 1980; Hall et al., 1982; Montesano et al., 1983; Montesano and Orci, 1985; Barcellos-Hoff et al., 1987). Studies with kidney epithelial cells revealed a potential role for laminin and laminin receptors in establishing epithelial polarity and tubulogenesis (Klein et al., 1988; Ekblom et al., 1990; Sorokin et al., 1990).

Although a previous study showed that the MDCK kidney cell line exhibits cell surface collagen binding activity (Salas et al., 1987), no putative collagen receptors were identified. More recent studies of integrin expression on MDCK cells revealed three integrins, $\alpha 1\beta 1$, $\alpha 2\beta 1$ and $\alpha 3\beta 1$, that may function as collagen or collagen/laminin receptors (Schoenenberger et al., 1994; Ojakian and Schwimmer, 1994; Spong and Garrod, 1994). However, the molecular basis of collagen-
MDCK cell interactions and their role in the morphogenetic capacity of MDCK cells remains unknown.

In this study, we identified the α2β1 integrin (Santoro et al., 1995), as a collagen receptor on MDCK cells. To further assess the function of the α2β1 integrin in not only the adhesive behavior of MDCK cells, but also to define its role in tubulogenesis and cyst formation, we inhibited expression of the α2β1 integrin using antisense RNA (Colman, 1990; Neckers and Whitesell, 1993; Keely et al., 1995). Studies with the antisense RNA expressing transfectants confirmed the role of the integrin as a collagen receptor on MDCK cells and revealed that α2β1 integrin-mediated interactions of the cells with the collagen matrix are key components of the morphogenetic program initiated by HGF/SF, and are essential for MDCK cell survival. The results are a striking demonstration of the interplay of a soluble growth factor and the ECM in mediating a complex morphogenetic event.

MATERIALS AND METHODS

Cell culture

MDCK cells were obtained from ATCC # CRL 6253, passage # 64. Cells were cultured in Dulbecco’s MEM with 10% fetal bovine serum (FBS) (HyClone, Logan, UT) and in the presence of 100 units/ml penicillin/streptomycin/gentamycin. Cells were routinely fed at 3-4 day intervals.

Adhesion

Experiments were performed in quadruplicate using 96-well plates (Immulon-2, Dynatech Laboratory, Chantilly, VA) coated with the indicated concentrations of substrate. Coating was performed for 16 hours at room temperature. Collagen type I (calliskin, Sigma, St Louis MO) and collagen type IV (human placenta, Sigma, St Louis MO) were dissolved in 50 mM acetic acid. Vitronectin (Gibco BRL, Grand Island, NY) was dissolved in TBS. Plates were subsequently blocked with 0.5% BSA (clinical reagent grade, ICN Biochemicals, Cleveland, OH) in Hanks’ balanced salt solution (HBSS; 136.9 mM NaCl, 5.4 mM KCl, 4.2 mM NaHCO3, 0.4 mM KH2PO4, 0.3 mM Na2HPO4, pH 7.2, and 5.5 mM D(+)-glucose). At 18 hours and 3 hours prior to the assay, cells were trypsinized and resuspended at 80-90% confluency. Cells for adhesion assays were harvested by treatment with phosphate buffered saline, pH 7.4, 0.5 mM Na2EDTA for 10-15 minutes. Cells were collected by centrifugation and washed 2 times with HBSS. Cells were resuspended in HBSS containing the appropriate divalent cations. In screening transfectants for adhesion to collagen type I, 300 μM MgCl2 was used as a suboptimal concentration to obtain maximum sensitivity. Typically, 15,000 cells/well were added and allowed to adhere for 15 minutes at 37°C. Wells were washed 6 times with HBSS after which cell adhesion was quantitated by the colorimetric cell adherence assay described (Landegren, 1984).

Morphogenetic assays

Cysts were clonally grown (Hall et al., 1982; McAteer et al., 1987) from single cells seeded at 600 cells/cm² on a 3 mm thick layer of hydrated rat tail collagen type I (Collaborative Biomedical Products, Bedford, MA). Collagen gels were prepared by neutralizing 1 volume of ice-cold collagen with 1 volume of 2 volume of Hepes buffered salt solution (1×: 25.2 mM Hepes, 81.3 mM NaCl, 5.3 mM KCl, 44.1 mM NaHCO3, 0.8 mM Na2HPO4, 5.5 mM D(+)-glucose). Standard medium was added to yield a final collagen concentration of 1.35 mg/ml and this solution was allowed to gel in a T75 flask. Cells were allowed to settle and adhere for 30 minutes after which medium was carefully removed and a second layer of neutralized collagen was used to overlay the cells. The collagen sandwich was then overlaid with standard medium which was replaced every 4 days. Cysts were grown for 7 days. Control medium or medium containing 0.5 volume hepatocyte growth factor (HGF/SF) in the form of 10-14-day-old 3T3 fibroblast-conditioned medium (Montesano et al., 1991a,b), supplemented with a standard concentration of penicillin/streptomycin/gentamycin and G-418, was added to induce tubulogenesis. Selected experiments were performed with recombinant HGF/SF (Collaborative Biomedical Products, Bedford, MA). Tubular structures were formed within 2 days of the addition of HGF/SF and a mature extensive branching network of tubules was observed after 4-5 days.

Chromatography

Collagen affinity chromatography was essentially performed as described (Santoro et al., 1988; Staatz et al., 1989). Collagen type I was dialyzed against 20 mM NaHPO4, pH 8.5, and coupled to CNBr-activated Sepharose (Pharmacia, Piscataway, NJ) according to the manufacturer’s instructions. Cell lysates for chromatography were prepared by lysing cells in 1% octylglucoside/TBS, pH 7.4, in the presence of protease inhibitors. Before applying the sample to the collagen-Sepharose affinity column, MgCl2 was added to a final concentration of 2 mM. After extensive washing, proteins were eluted with 2 mM EDTA/TBS, pH 7.4/0.1% octylglucoside. After chromatography, fractionated samples were analyzed for α2-integrin subunit content by western blotting.

Western blot analysis

Reduced SDS solubilized lysates from 5×105 cells were analyzed on a 5% SDS-polyacrylamide gel and transferred onto a polyvinylidene difluoride membrane (Millipore Cont. Water Systems, Bedford, MA). Matched sample loading was confirmed on an identical Coomassie Brilliant Blue stained gel. Canine integrin α2-subunit protein was detected using a 1:2,500 dilution of a rabbit polyclonal directed against a synthetic peptide derived from the mouse integrin C-terminal α2-sequence (Wu and Santoro, 1994). A 1:2,000 dilution of a rabbit polyclonal directed against a synthetic peptide derived from the human integrin C-terminal α1-sequence (Menko and Philip, 1995) was used to detect the canine α1 integrin homologue in the cell lysates of parental MDCK, control pRSVneo and antisense RNA expressing cell lines. Subsequently, immunoreactive material was visualized using a 1:4,000 dilution of alkaline-phosphatase conjugated goat anti-rabbit polyclonal IgG and a substrate reaction by addition of NBT (0.32 mg nitro blue tetrazolium chloride/ml), BCIP (0.16 mg 5-bromo-4-chloro-3-indolyolphosphate/ml) in 100 mM Tris–HCl, pH 9.5, 100 mM NaCl, 5 mM MgCl2.

Constructs

Plasmid pRc/RSV (Invitrogen, San Diego, Ca) was used to generate control cell lines. The antisense construct pRSVα2 was created by ligation of a 1.3 kb XbaI–HindIII α2-integrin subunit cDNA fragment (Zutter et al., 1992) containing bases 1-1,296 of the published sequence (Takada and Hemler, 1989) into the multiple cloning site. Proper antisense orientation was confirmed by dedoxygenucleotide sequencing. Large scale isolation of the constructs was performed as described (Sambrook et al., 1989).

Transfection and clonal selection

40-70% subconfluent monolayers of cells were transfected with 25 μg/ml plasmid DNA (pRSVα2 or control pRSV) mixed with 35 μl/ml Lipofectin® (Gibco BRL, Grand Island, NY) according to the manufacturer’s instructions for transfection of adherent cells. After 20 hours, 1 volume of medium containing 20% FBS was added to the cells and incubated for 24 hours after which medium was removed and cells were grown in standard medium for another 24 hours. Subsequently, cells were trypsinized and reseded at subconfluent cell density in standard medium containing 750 μg/ml active ingredient.
G418 (Geneticin®, Gibco BRL, Grand Island, NY). After 3 weeks, G418 resistant cells were trypsinized and cloned using limited dilution.

**Labeling**

32P-labeled probes were generated by random priming (Sambrook et al., 1989). Templates used in these reactions were: a 1.3 kb human α2-integrin subunit cDNA fragment containing basepairs 1-1,296 (Zutter et al., 1992), a 0.7 kb GAPDH cDNA fragment for northern blot analysis, and a 0.73 kb pRSV fragment containing 0.13 kb of SV40 promotor and 0.6 kb of the neomycin-resistance gene for Southern blot analysis.

**Southern blot analysis**

Preparation of DNA from various cell lines was performed according to the proteinase K lysis method for eukaryotic cells (Davis et al., 1986). DNA was digested with the appropriate restriction enzymes, analyzed on a 0.8% agarose gel (10 μg/lane) and transferred to a nitrocellulose membrane (BA-85, Schleicher and Schuell, Keene, NH) in 20× SSPE (standard saline phosphate EDTA; 1×: 150 mM NaCl, 10 mM NaH2PO4·H2O, pH 7.4, 1 mM Na2EDTA). After baking and prehybridization (5× SSPE, 2× Denhardt, 0.1% SDS, 40% formamide, 100 μg/ml sonicated and denatured hsDNA), membranes were hybridized overnight at 42°C in fresh prehybridization solution to which 10% dextran sulfate and 32P-labeled probe, typically >2 cpm/ml, were added. Final washes were performed at 42°C in 0.5× SSPE/0.1% SDS and subjected to autoradiography.

**Northern blot analysis**

RNA from various cell lines was isolated by the guanidine/CsCl method (Sambrook et al., 1989). Total RNA (10 μg/lane) was analyzed on a 1% agarose gel in 20 mM MOPS/3.7% paraformaldehyde and transferred to a nitrocellulose membrane (BA-85, Schleicher and Schuell, Keene, NJ) in 20× SSC. After baking and prehybridization (5× SSC, 2× Denhardt, 0.1% SDS, 40% formamide, 100 μg/ml sonicated and denatured hsDNA), membranes were hybridized under similar conditions as described for northern blotting. Washes were performed at 42°C in 0.4× SSC/0.1% SDS final stringency for α2-integrin subunit mRNA and in 0.2× SSC/0.1% SDS final stringency for GAPDH mRNA detection and subjected to autoradiography.

**Apoptosis assays**

Control pRSVneo and pRSVα2' cells were cultured for 96 hours in three-dimensional collagen gels. The collagen gels were washed extensively with HBSS and fixed in 1% paraformaldehyde/PBS for 90 minutes at room temperature. Then the gels were extensively washed with PBS and incubated with 10 Kunitz units of DNAse-free RNase/ml PBS for 90 minutes at 37°C. Subsequently, gel fragments were isolated, washed with PBS, applied on a Superfrost R/Plus slide (Fischer, Pittsburgh, PA) and stained with 5 μg/ml propidium iodide (Sigma, St Louis, MO) in PBS for 30 minutes at room temperature. After application of Vectashield® (Vector Laboratories, Burlingame, CA) and a coverslip, the gels were examined by immunofluorescence. Apoptotic cells exhibited the characteristic appearance of cell-bodies with membrane blebbing and with shrunken, condensed and fragmented nuclei (Wyllie et al., 1980).

Low molecular mass DNA from trypsinized adherent cells, seeded initially on a hydrated layer of collagen type I and cultured for 3 days, was extracted with 10 mM Tris-HCl, pH 7.4, 10 mM Na2EDTA, 0.5% Triton X-100, phenol/chloroform extracted and ethanol precipitated. Samples were treated with DNase-free RNase for 30 minutes at 37°C. Subsequently, DNA samples reflecting equivalent numbers of cells (2×10^6 cells/lane) were loaded on a 1.5% agarose gel in TAE buffer. Apoptosis was indicated by the characteristic ladder of DNA fragmentation.

**RESULTS**

**Identification of α2β1 integrin as a collagen receptor on MDCK cells**

MDCK cells adhered to collagen types I and IV in a divalent cation dependent manner (Fig. 1A). Adhesion was supported by Mg2+, but not by Ca2+ or EDTA-containing medium in a manner similar to that previously established for the α2β1 integrin (Staatz et al., 1989). The presence of the canine α2β1 integrin homologue on MDCK cells was revealed by western blotting with a polyclonal antibody, directed against the murine α2-integrin subunit (Fig. 1B). Immunofluorescent labeling of MDCK cells grown as a monolayer showed that this integrin had a basolateral distribution (data not shown). Furthermore, the integrin could be isolated by collagen-Sepharose affinity chromatography carried out in the presence of 2 mM Mg2+ (Fig. 1B) whereas no collagen-binding activity could be detected in the presence of Ca2+ (data not shown). Fig. 1B shows the result of a typical experiment. Lane 1 shows the α2-integrin protein content in the starting cell lysate. After chromatography, no α2-integrin positive material was detected in

![Fig. 1. Characterization of collagen binding activity on MDCK cells.](image-url)

(A) Divalent cation dependent adhesion of MDCK cells to collagen type I (filled bar) and IV (hatched bar). Adhesion was determined in the presence of 100 μM EDTA, 2.0 mM Ca2+, or 2.0 mM Mg2+. Values are mean ± s.d., obtained in a typical experiment (n=3) performed in quadruplicate. (B) Western blot analysis of Mg2+-dependent collagen affinity chromatography fractions using a polyclonal against mouse α2-integrin subunit. Lane 1, MDCK cell lysate starting material (St); lane 2, unbound flowthrough (Fl); lane 3, material bound in a Mg2+-dependent manner subsequently eluted with EDTA (El). Arrowheads show positions of 215 kDa and 105 kDa protein standards.
the flowthrough fractions (lane 2), but all \( \alpha_2 \)-integrin protein was detected in the EDTA eluted fractions (lane 3).

**Transfection and characterization of antisense constructs**

The above studies established the presence and collagen binding activity of the \( \alpha_2 \beta_1 \) integrin on MDCK cells. Because inhibitory antibodies directed against the canine homologue of \( \alpha_2 \beta_1 \) integrin do not exist, the traditional use of such antibodies to assess the role of the integrin in adhesion and, more importantly, collagen-induced morphogenesis was precluded. In order to perturb \( \alpha_2 \beta_1 \) integrin expression during branching morphogenesis and cyst formation, we chose to inhibit expression by using antisense RNA. A 1.3 kb human \( \alpha_2 \)-integrin subunit cDNA fragment was cloned in the antisense orientation into a eukaryotic expression vector under transcriptional control of the RSV-promotor. The construct was transfected in MDCK cells. After G418-selection and cloning by limited dilution, 30 G418-resistant clones were screened for \( \alpha_2 \)-integrin protein expression and for their ability to adhere to collagen type I under \( \text{Mg}^{2+} \)-dependent conditions. Adhesion to collagen and \( \alpha_2 \)-integrin protein expression of most G418-resistant clones was affected by the antisense RNA expressing construct. Fig. 2 shows a representative matched screening of

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**Fig. 2.** Screening of antisense RNA expressing transfectants. Transfectants were analyzed for adhesion to collagen and \( \alpha_2 \)-integrin subunit expression by western blotting. Results of adhesion assay and reflectance-scanning of blotted \( \alpha_2 \)-subunit protein were normalized to control values.

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**Fig. 3.** Characterization of antisense RNA expressing cell lines. (A) Southern blot analysis of \( \text{EcoRI} \)-digested DNA probed with a neomycin resistance gene specific-fragment, demonstrating the presence of stably integrated pRSV constructs. From left: lane 1, MDCK-non transfected cells; lane 2, pRSVneo control cell line; lane 3, clone pRSV\( \alpha_2 \) #7; lane 4, clone pRSV\( \alpha_2 \) #8; lane 5, clone pRSV\( \alpha_2 \) #23. Arrowheads show \( \lambda/HindIII \) marker positions. (B) Northern blot analysis of pRSVneo and pRSV\( \alpha_2 \) antisense constructs total RNA, probed with \( \alpha_2 \)-integrin subunit cDNA and GAPDH cDNA. From left: lane 1, clone pRSV\( \alpha_2 \) #7; lane 2, clone pRSV\( \alpha_2 \) #8; lane 3, clone pRSV\( \alpha_2 \) #23; lane 4, pRSVneo control cell line. (C) Western blot analysis of total protein lysates using a polyclonal antiserum against mouse \( \alpha_2 \)-integrin subunit. From left: lane 1, pRSVneo control; lane 2, clone pRSV\( \alpha_2 \) #7; lane 3, clone pRSV\( \alpha_2 \) #8; lane 4, clone pRSV\( \alpha_2 \) #23. Arrowheads show positions of 215 kDa and 105 kDa protein standards. (D) Western blot analysis of total protein lysates using a polyclonal antiserum against human \( \alpha_1 \)-integrin subunit. From left: lane 1, parental MDCK control; lane 2, pRSVneo control; lane 3, clone pRSV\( \alpha_2 \) #7; lane 4, clone pRSV\( \alpha_2 \) #8; lane 5, clone pRSV\( \alpha_2 \) #23. Arrowheads show positions of 214 kDa and 111 kDa protein standards.
11 clones for their ability to adhere to collagen type I and \( \alpha_2 \)-integrin protein expression. Based on these screenings, clones pRSV\( \alpha_2 \) #7 and #23 which exhibited low level adhesion and \( \alpha_2 \)-integrin protein expression, and clone pRSV\( \alpha_2 \) #8, exhibiting intermediate levels, were selected for further characterization.

Transfection and integration of the constructs into the genome was confirmed by Southern blot analysis which revealed integration at distinct sites in each of the clones (Fig. 3A). Analysis of \( \alpha_2 \)-integrin subunit mRNA expression by northern blot analysis revealed striking decreases in the \( \alpha_2 \)-integrin subunit transcript in clones pRSV\( \alpha_2 \) #7 and #23 and a more modest decrease in clone pRSV\( \alpha_2 \) #7 after normalization to GAPDH mRNA (Fig. 3B). The decreases in the \( \alpha_2 \)-integrin subunit transcript were accompanied by more dramatic decreases in \( \alpha_2 \)-integrin subunit protein expression. Western blot analysis of cell lysates from the clones showed that \( \alpha_2 \)-integrin subunit protein expression was markedly decreased (Fig. 3C). Densitometric scanning of the blots revealed a 70-80% decrease for clones pRSV\( \alpha_2 \) #7 and #23 and a 40% decrease for clone pRSV\( \alpha_2 \) #8 as compared to the \( \alpha_2 \)-integrin subunit protein expression in the control transfectant clone pRSVneo. Western blot analysis of \( \alpha_1 \)-integrin protein expression of control and antisense RNA expressing cells revealed no changes in expression of this integrin, therefore indicating that the \( \alpha_2 \)-integrin protein expression was specifically suppressed by the antisense construct.

**Effects on cell adhesion**

Fig. 4A,B depicts the Mg\(^{2+}\)-dependent adhesion of control clone pRSVneo and antisense clones pRSV\( \alpha_2 \) #7, #8 and #23 to collagen types I and IV. Antisense clones pRSV\( \alpha_2 \) #7 and #23, which express low levels of \( \alpha_2 \)-integrin protein, exhibited only low level adhesion to collagen types I and IV as compared to control clone pRSVneo. Adhesion of clone pRSV\( \alpha_2 \) #8, which expresses an intermediate level of \( \alpha_2 \)-integrin protein, to collagen type I was only slightly decreased and adhesion to collagen type IV was moderately decreased. Adhesion of non-transfected MDCK cells was similar to that of the control clone pRSVneo (data not shown). In contrast to the results obtained for collagen, all cell lines showed similar adhesive activity to control vitronectin substrates (Fig. 4C). These data, therefore, provide additional, functional evidence for the specificity of the antisense construct.

**Effects on morphogenesis**

In order to assess the role of the \( \alpha_2\beta_1 \) integrin in cyst formation and tubulogenesis, the morphogenetic collagen overlay assay was employed. Clonal growth of control clone pRSVneo cells for 7 days resulted in spheroidal cyst formation (Fig. 5A). No differences were observed between cysts formed by non-transfected MDCK cells or control transfectants. In contrast, small rudimentary cyst formation was observed when antisense RNA expressing clones were used. Both the number and size of cysts were profoundly reduced (Fig. 5B,C,D). Cells of clone pRSV\( \alpha_2 \) #23 sporadically formed rudimentary cysts while most of the seeded cells died in the single cell stage (day 1-2 in cyst formation). Similar though less extensive cell death was observed for clones pRSV\( \alpha_2 \) #7 and #8. No further growth of the surviving small cysts was observed after day 5-7. Cyst size remained considerably less than control.

Tubulogenesis was induced by adding 0.5 volume of 3T3 fibroblast-conditioned medium to 7-day-old cyst cultures. A rapid and vigorous response to the added HGF/SF by the
Fig. 5. Role of α2β1 integrin in morphogenesis. Clonal growth of control pRSVneo cells in collagen gels for 7 days resulted in spheroidal cyst formation (A). Experiments (n=4) were performed in duplicate. Only small cyst formation was observed when antisense expressing pRSVα2' #7, #8 and #23 cells were used (B, C, D). Tubulogenesis was induced by adding 3T3 fibroblast-conditioned medium, as a source of HGF/SF, to 7-day-old cyst cultures. An extensive branching network was observed after 4 days (E). In contrast, the small preformed cysts formed by antisense clones pRSVα2' #7 and #23 completely failed to branch in response to the HGF/SF-containing medium (F, H), although initial spiking was observed, and the cysts increased in size. Cysts derived from clone pRSVα2' #8 showed an occasional short branching arm (G, arrow). Bar, 100 μm.
Branching morphogenesis of MDCK cells

preformed cysts of control clone pRSVneo was observed within 1 day, which entailed extensive pseudopod formation and initial branching. An extensive branching network was established within 4-5 days. No apparent change of the structures was observed after this time (Fig. 5E). No difference in the branching morphogenesis was observed between preformed cysts derived from non-transfected parental MDCK or pRSVneo transfected cells. In contrast, the small cysts formed from antisense clones pRSVα2’ #7 and #23 completely failed to branch in response to the HGF/SF-containing medium (Fig. 5F,H). Similar results were obtained when control pRSVneo cells and pRSVα2’ #23 cells were stimulated with 25 ng/ml rHGF (data not shown). Cysts derived from clone pRSVα2’ #8 occasionally showed some primitive branching (Fig. 5G). Typically only one/two short branching arms were observed (in case of clone pRSVα2’ #8) that remained small in size and primitive in appearance as compared to control. However, prolonged exposure (up to 2-3 weeks) to the HGF-containing medium did stimulate the growth of these preformed small cysts and resulted in large flat, macroscopically visible cysts (>1 mm) but in no case was branching observed (Fig. 6A,B).

Effects on survival
As described above, cells of the pRSVα2’ clones exhibited reduced survival in three-dimensional collagen gels and formed rudimentary cysts. Survival of cells in collagen gels was evaluated by counting dead and alive cells in 3-5 fields (1 cm²). More than 98% of the cells of clone pRSVα2’ #23 died in the single cell stage within 24-48 hours while only 1% of pRSVneo control cells died. Similar though less extensive cell death, ranging from 71-86%, was observed for pRSVα2’ #7 and #8 cells. To confirm that cells died through an apoptotic mechanism, cells were stained with propidium iodide to visualize nuclei and genomic DNA. Fig. 7A, shows that pRSVα2’ #23 cells are shrunken cells with condensed, fragmented, genomic DNA indicative of apoptosis (d-g). Staining of pRSVneo control cells (a-c) shows single cells with intact nuclei and formation of a small cluster of viable cells.

In addition, antisense RNA expressing cells were also shown to undergo apoptosis when seeded on top of a hydrated layer of collagen type I. DNA-ladder analysis (Fig. 7B) shows that antisense pRSVα2’ #7, pRSVα2’ #8 and pRSVα2’ #23 cells (lanes b-d) but not pRSVneo control cells (lane a) underwent genomic fragmentation characteristic of apoptotic cell death.
DISCUSSION

The purpose of this study was to identify the contribution of epithelial cell-collagen interactions to kidney tubulogenesis and cyst formation. To do so, we exploited the morphogenetic potential of MDCK cells to form cysts in hydrated gels of collagen type I (Hall et al., 1982; McAteer et al., 1987; Montesano et al., 1991a,b). The cysts were induced to form an extensive branching tubular network by stimulation with HGF/SF (Montesano et al., 1991a,b). Lumen formation and in some cases tubulogenesis also occurs when other kidney epithelial cells are cultured in gelled collagen type I or reconstituted basement membrane (Wohlwend et al., 1985; Taub et al., 1990; Perantoni et al., 1991).

Cell-matrix interactions are complex in nature. In recent years, our understanding of these interactions has considerably advanced with the identification of a family of extracellular matrix receptors, the integrins (Albelda and Buck, 1990; Hynes, 1992). Immunohistochemical studies of the developing and adult nephron (Korhonen et al., 1990; Simon and MacDonald, 1990) as well as studies of several kidney cell lines have established patterns of integrin expression. Within the kidney, expression of the α2β1 integrin collagen receptor is largely restricted to cells of the distal tubules and collecting ducts whereas other putative collagen receptors like the α1β1 and α3β1 integrin collagen/laminin receptors have a wider distribution. These three collagen/laminin integrin receptors have also been detected on MDCK cells (Schoenenberger et al., 1994; Ojakian and Schwimmer, 1994; Spong and Garrod, 1994). The high level of α2β1 integrin expression by MDCK cells is consistent with their derivation from distal tubules or collecting ducts (Leighton et al., 1970; Ojakian et al., 1987; Birchmeier and Birchmeier, 1993). Both Schoenenberger et al. (1994) and Ojakian and Schwimmer (1994) showed that cross-reactive anti-β1 integrin monoclonal antibodies inhibited divalent cation-dependent adhesion of MDCK cells to collagens. The cross-reactive anti-β1 integrin monoclonal antibodies also blocked collagen-induced reversal of polarity by MDCK cells (Zuk and Matlin, 1994; Ojakian and Schwimmer, 1994; Spong and Garrod, 1994). These findings are consistent with a putative role for the α1β1, α2β1 and/or the α3β1 integrin as collagen receptors on MDCK cells.

Our results confirm that MDCK cells express the α2β1 integrin and in addition establish that these cells use the α2β1 integrin as a collagen receptor. Since no (cross-reactive) inhibitory antibodies directed against the canine α2-integrin subunit exist, we used an antisense RNA approach to decrease α2β1 integrin expression on MDCK cells to investigate its function in both adhesion and complex morphogenetic events. The use of the antisense RNA approach to probe receptor function avoids some pitfalls recently recognized in the use of inhibitory antibodies whereby antibodies directed against one integrin indirectly result in the functional inactivation of a second integrin (Blystone et al., 1994).

Analysis of antisense RNA expressing clones by northern and western blotting revealed decreased α2-integrin subunit expression at both the mRNA and protein levels. In general, the magnitude of the decreases in mRNA and protein were concordant. Since antisense RNA may act by several mechanisms (Colman, 1990; Neckers and Whitesell, 1993), it is not imperative that the decrease in mRNA be of the same magnitude as the decrease in protein. In cases of discordance, mechanisms acting at the posttranscriptional level appear to be dominant. Profound changes in protein expression have been achieved with little or no effect on mRNA level (Colman, 1990; Neckers and Whitesell, 1993; Keely et al., 1995). Decreased α2β1 integrin expression on MDCK cells resulted in markedly diminished Mg2+-dependent adhesion to collagen types I and IV. Apparently, there is no large excess of receptors present so that even modest decrements in receptor expression have significant effects on adhesion. We have recently made similar observations with T-47D breast epithelial cells (Keely et al., 1995). We conclude that α2β1 integrin expression is a major determinant of MDCK cell adhesion to collagen. The suppression of α2β1 integrin appeared specific as no decreases in α1β1 integrin collagen receptor expression were detected. In addition, functional studies showed that adhesion to vitronectin substrates was unaffected.

Loss of α2β1 integrin expression also profoundly reduced the viability and the morphogenetic capacity of MDCK cells cultured in collagen gels. When the antisense RNA expressing cells were seeded in or on top of three-dimensional collagen gels significant apoptotic cell death was observed. We have also observed increased apoptotic cell death of antisense RNA expressing cells on their endogenous collagen-rich ECM (data not shown) which is rapidly produced and deposited by MDCK cells under standard culture conditions (Salas et al., 1987; Wang et al., 1990a). Although, it has previously been shown that MDCK cells will undergo apoptosis when all cell matrix interactions are perturbed (Frisch and Francis, 1994), our findings provide the first data supporting a specific role for α2β1 integrin-dependent anchorage in maintenance of MDCK cell survival on and in collagen gels. These data add to a growing body of evidence in support of the critical role of integrin-matrix interactions as determinants of cell survival (Meredith et al., 1993; Ruoslahti and Reed, 1994; Bates et al. 1994; Montgomery et al., 1994; Boudreau et al. 1995).

The antisense RNA expressing cells, that did survive in the collagen gel formed small cysts, but did not undergo tubule formation and branching morphogenesis upon subsequent exposure to HGF/SF. However, the small cysts did dramatically increase in size in response to the growth factor indicating that the growth response of the transfectants to HGF/SF is intact. These findings thus implicate both α2β1 integrin-mediated interaction with collagen and signals derived from the interaction in the complex morphogenetic processes of cyst and tubule formation. As noted above, MDCK cells rapidly synthesize and deposit ECM components like collagen type IV, laminin and proteoglycans leading to the accumulation of matrix around the cells. The resulting ECM is more complex in nature than the simple type I collagen matrix present at the beginning of the experiment. In addition, collagen-binding adhesive serum proteins such as vitronectin and fibronectin may further increase the complexity of the matrix. Therefore, the failure of the MDCK cell antisense transfectants to undergo morphogenesis in the resulting complex matrix emphasizes the importance of the role of α2β1 integrin-collagen interactions.

The role we find for the α2β1 integrin in morphogenesis is consistent with other recent studies. The formation of breast ductule-like structures in collagen gels by mammary epithelial cells is abrogated by disruption of α2β1 integrin function using either inhibitory antibodies or expression of antisense RNA
(Keely et al., 1995). Berdechevski et al. (1992) showed that anti-α2 integrin antibodies inhibited 'rapid' morphogenesis of mammary epithelial cells induced by particulate collagen. Conversely, re-expression of the α2β1 integrin in an α2β1 integrin negative, poorly differentiated, breast carcinoma cell line resulted in the gain of morphogenetic capacity in three-dimensional ECM gels (Zutter et al., 1995).

The results obtained in our morphogenetic experiments suggest that even a moderate decrease in α2-integrin subunit protein expression, as observed with pRSVα2/β1-#8, results in the almost complete failure to branch. Presumably, the number of receptors needed for signaling and/or subsequent adhesive interactions during migration through the collagen gel, is too low. Other pRSVα2/β1 cell lines with normal α2-integrin protein expression, exhibited normal branching morphogenesis. Initial spiking of the enlarging cysts derived from the antisense clones was observed after exposure to HGF/SF. In the case of clone pRSVα2/β1-#8, formation of a few short primitive branching arms was sometimes observed. These observations imply that at least some of the initial signaling pathways of HGF/SF are still intact. pp60-src activity, which is modulated by the interaction of the HGF/SF with its receptor c-met (Ponzetto et al., 1994), has been shown to be involved in MDCK cyst deformation and spiking of individual cyst cells (Warren and Nelson, 1987; Warren et al., 1988).

It, thus, seems clear that at least two determinants acting in concert, HGF/SF and the α2β1 integrin collagen receptor, are involved in normal MDCK cell morphogenesis. Interestingly, upon prolonged exposure to HGF/SF, the small preformed cysts of the antisense clones formed large cysts. The large cysts are probably a consequence of both the high collagenase and mitogenic activities induced by HGF/SF (Montesano et al., 1991a,b) in combination with the loss of essential cell-collagen interactions that support tubulogenesis and branching. These same two determinants may also play a role in pathological cyst formation. Injury models have shown that integrin-ECM interactions are disrupted when tubules are damaged. Injury is followed by integrin re-distribution/re-expression (Goligorsky et al., 1993) and temporally elevated levels of HGF/SF during renal tubule repair (Igawa et al., 1993; Kawaida et al., 1994). The repair mechanisms involve tubular cell proliferation balanced by apoptotic cell death of surplus cells (Coles et al., 1993; Nouwen et al., 1995). Therefore, chronic damage to the α2β1 integrin expressing renal architecture, such as the distal tubules or collecting ducts, might result in disruption of integrin-collagen or laminin interactions, with continuous exposure to HGF/SF leading to cyst formation.

In conclusion, we have successfully employed an antisense RNA technique to ascertain the role of the α2β1 integrin in MDCK cell morphogenesis. Our results showed that ligation of this integrin collagen receptor is required for normal cyst formation, for elongation and branching morphogenesis of MDCK cells in three-dimensional collagen gels which occurs in response to HGF/SF, and for MDCK cell survival in collagen matrices. The role of other putative collagen receptors on MDCK cells remains to be established.

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REFERENCES
subunit of integrins are characteristically expressed in distinct segments of the developing and adult human nephron. J. Cell Biol. 111, 1245-1254.


