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Integrin α6Bβ1 is involved in kidney tubulogenesis in vitro

Mats Falk[1], Katriina Salmivirta[1], Madeleine Durbeej[1], Erik Larsson[1], Marja Ekblom[1], Dietmar Vestweber[2] and Peter Ekblom[1,*]

[1]Departments of Animal Physiology and Pathology, Uppsala University, Biomedical Center, Box 596, Husargatan 3, S-75124, Uppsala, Sweden
[2]Max-Planck-Institut für Immunbiologie, Freiburg, Germany

*Author for correspondence

SUMMARY

Laminin-1 has previously been shown to be of major importance for the development of kidney tubules. Antibodies against fragments E8 and E3 of laminin-1 perturb kidney development in vitro. We here studied expression of integrins α6β1 and α6β4, two known laminin receptors, during kidney development. Integrin β1 subunit could be detected by immunofluorescence on all cell types of embryonic mouse kidney, but we could not detect integrin β4 subunit in embryonic kidney by immunofluorescence or by in situ hybridization. The presence of integrin α6 subunit in all epithelia of embryonic kidney was demonstrated by immunofluorescence and by in situ hybridization. RT-PCR showed that α6B is the major splice variant in embryonic kidney. During in vitro conversion of nephrogenic mesenchyme to epithelial tubules, a strong increase in the expression of the 6 kb mRNA for α6 integrin subunit was seen by northern blotting at the onset of epithelial morphogenesis, on day two of culture. Immunoprecipitation of extracts from embryonic kidney with antibodies against α6 subunit yielded bands corresponding to the expected size of β1 integrin subunit but not of β4 subunit. Monoclonal antibodies against either α6 or β1 subunit but not against E-cadherin blocked kidney tubulogenesis in vitro. This suggests that integrin α6Bβ1 is involved in kidney tubulogenesis in vitro. Another possibility is that the antibodies against integrin α6 and β1 subunit cause abnormal signalling by the integrin.

Key words: Laminin, Integrin, Epithelium, Kidney, Apoptosis

INTRODUCTION

The formation of polarized epithelial cells is a fundamental morphogenetic process during embryogenesis. In most parts of the body, epithelial cells form tube-like structures, and it is of major importance to understand how such tubes form during embryogenesis (Ekblom et al., 1986; Rodriguez-Boulan and Nelson, 1989; Matlin and Caplan, 1992). The first epithelium to form in mammals is the trophectoderm. Antibody perturbation and gene knock-out experiments have established a crucial role for E-cadherin in the development of the trophectoderm (Takeichi, 1991; Kemler, 1993; Larue et al., 1994). The formation of kidney tubules resembles the formation of the trophectoderm (Ekblom et al., 1986). In the embryonic kidney, non-polarized mesenchymal cells convert into a new epithelium, and begin to express epithelial markers such as E-cadherin (Grobstein, 1956; Vestweber et al., 1985). Yet, antibodies against E-cadherin have failed to perturb kidney tubulogenesis in vitro (Vestweber et al., 1985), suggesting that other cadherins, other cell adhesion molecules or cell-matrix interactions are required for the development of kidney tubules.

Our previous studies on laminin have suggested a major a role for cell-matrix interactions in tube formation. Laminin-1 is a cell adhesive glycoprotein of basement membranes. It is composed of three chains, α1, β1 and γ1 (Burgeson et al., 1994; Timpl and Brown, 1994). These chains appear sequentially during kidney tubule development, and laminin α1 chain is a good marker for onset of epithelial cell polarity during conversion of mesenchyme to epithelium (Ekblom et al., 1990). Antibodies against two fragments of laminin-1, E8 and E3, perturb the formation of kidney tubules in organ culture (Klein et al., 1988) suggesting that specific receptors for these two fragments are important for kidney tubulogenesis.

Recently, it was suggested that the E3 fragment of laminin-1 in developing kidney tubules binds to the dystroglycan complex. The expression of dystroglycan mRNA was studied in considerable detail by northern blotting and in situ hybridization. Dystroglycan was found to be coexpressed with its putative ligand, the E3 fragment, during kidney tubule development (Durbeej et al., 1995). Less is known about receptors for the E8 fragment in this system, although the presence of integrins containing the α6 subunit on embryonic kidney tubules was demonstrated several years ago (Korhonen et al., 1990b; Sorokin et al., 1990). The E8 fragment is known to bind to the α6β1 integrin in other cells (Sonnenberg et al., 1990a,b; Aumailley et al., 1990; Timpl and Brown, 1994). Immunofluorescence analysis of organ cultures of embryonic kidney mesenchyme suggest that the integrin α6 subunit coappears with laminin α1 chain during conversion of mesenchyme to epithe-

lium, and antibodies against the integrin α6 subunit perturb kidney tubulogenesis in vitro (Sorokin et al., 1990). By analogy to other systems (Sonnenberg et al., 1990b) it has therefore been proposed that the E8 fragment binds to integrin α6β1 in the developing kidney (Ekblom, 1992, 1993). Yet, the association of the α6 subunit with the β1 subunit in the embryonic kidney has never been demonstrated, and the available evidence for the presence of the α6 subunit on embryonic kidney tubules is based exclusively on immunofluorescence.

The integrin α6 subunit is known to associate with either the β1 or β4 subunit to form the laminin receptors α6β1 and α6β4 (Sonnenberg et al., 1990b, 1993; Lee et al., 1992; Niessen et al., 1994; Spinardi et al., 1995). These two laminin receptors may codistribute in some tissues and cells (Sonnenberg et al., 1990a; Tamura et al., 1990; Natali et al., 1992). Expression of α6β4 integrin seems to be a typical feature of stratified squamous epithelia (Sonnenberg et al., 1991) and endothelium of medium-sized blood vessels (Kennel et al., 1992), but it is also expressed in some other epithelial structures. Both receptors show a strong polarization towards the basal side of epithelial cells, implicating interactions with epithelial basement membrane components. In cells where all three integrin chains are present (α6β1 and β4), the α6 subunit preferentially associates with β4 (Tözeren et al., 1994). It is likely that the β4 integrin subunit is poorly expressed in the adult kidney (Korhonen et al., 1990a), but Natali et al. (1992) reported the presence of some integrin β4 subunit in the kidney. In an established kidney cell line the integrin α6β4 was also detected (Schoenenberger et al., 1994). Since the β1 and β4 subunits have very different intracellular domains, they might affect cells in distinct ways.

In the current study, we have therefore studied the chain association of the α6 subunit in embryonic mouse kidney by immunoprecipitation. The presence of integrin subunits β1 and β4 was also studied by immunofluorescence and for the β4 integrin subunit the data were verified by in situ hybridization. Furthermore, the expression of α6 subunit was studied by northern blotting during conversion of mesenchyme to epithelium in vitro, and during in vivo development both by immunofluorescence and by in situ hybridization. Two intracellularly different α6 splice variants have been demonstrated (Hogervorst et al., 1991), and they may have distinct functions (Shaw et al., 1995). Therefore, we have also studied the expression of these variants in the embryonic kidney by RT-PCR. Finally, functional studies were performed in an organ culture system using blocking antibodies against integrin α6 (Sonnenberg et al., 1990a; Kadoya et al., 1995), integrin β1 subunit (Lenter et al., 1993), and E-cadherin (Vestweber and Kemler, 1985).

MATERIALS AND METHODS

Antibodies

Integrin α6 subunit was detected with rat monoclonal antibody (mAb) GoH3 (Sonnenberg et al., 1990a), purchased from Immunotech SA, Marseille, France. Integrin β1 subunit was detected with rat mAb 9EG7 (Lenter et al., 1993), or with rabbit antiserum against β1 integrin (Gullberg et al., 1989) provided by Dr Donald Gullberg, Department of Animal Physiology, Uppsala University. Rat mAbs against integrin β4 subunit were gifts from Dr Stephen Kennel, Oak Ridge Laboratory, Oak Ridge, Tennessee, USA (3446-11A; Kennel et al., 1989) and Dr Pier Carlo Marchisio, Torino University (SP 90395). E-cadherin was detected with mAb DECMA (Vestweber and Kemler, 1985). L-9393, a rabbit polyclonal antibody recognizing laminin-1, was purchased from Sigma Immunochemicals (St Louis, MO).

Organ culture

Kidneys were dissected from 12-day NMRI mouse embryos. The day of appearance of the vaginal plug was designated day 0. Nuclepore filters were put onto a plastic grid, and preincubated with 0.1% bovine serum albumin (BSA) in water to prevent antibody binding to plastic. Embryonic kidneys were cultured on filters at 37°C, 5% CO_2 in improved minimum essential medium, zinc option (IMEM- ZO) supplemented with 10% fetal calf serum (FCS) and 1% L-glutamine (Grobstein, 1955; Vestweber et al., 1985). For functional studies, blocking mAbs GoH3, 9EG7 and DECMA were added to the culturing medium at onset of culture. Differentiation in vitro was followed for 3 days. Explants were photographed and evaluated for number of tubules and condensates (Durbeej et al., 1995). Transfilter cultures of isolated mesenchyme and inducer tissue were performed as described (Grobstein, 1956; Saxén et al., 1968; Vestweber et al., 1985) and RNA was isolated from the mesenchymes (Ekblom et al., 1990).

Immunofluorescence and histology

For immunofluorescence kidneys were frozen in Tissue Tek (Miles, Naperville, IL) using liquid nitrogen. Sections (6 μm) were fixed with acetone for 2 minutes, washed with phosphate buffered saline (PBS) and incubated with 1% BSA in PBS for 10 minutes to block unspecific binding. Primary antibodies were: GoH3 (1 μg/ml), anti-β1 serum (2 μg/ml), 346-11A (2 μg/ml), SP 90 395 (3 μg/ml) and L-9393 (1:500). Bound primary antibodies were visualized using secondary antibodies (goat anti-rat or goat anti-rabbit) coupled with either FITC or Cy-3 (Jackson Immunoresearch Laboratories, West Grove, PA). For histology cultured kidneys were fixed in Bouin solution for 24 hours and washed several times in 70% ethanol. Paraffin embedded organs were cut and sections were stained with Hematoxylin-Eosin. Micrographs were taken using a Zeiss Axiophot microscope.

Immunoprecipitation and SDS-PAGE

For each immunoprecipitation approximately 50 to 70 embryonic day 15 kidneys were dissected and metabolically labelled for 4 hours at 37°C with [^{35}S]methionine and -cysteine (1 mCi/ml) in RPMI-1640 medium, without methionine and cysteine, supplemented with 1 mM Hepes buffer. Solubilisation was done by homogenisation in lysis buffer (0.1% SDS, 1% Triton X-100, 1% deoxycholate, 0.65 mM $MgSO_4$, 1.22 mM $CaCl_2$ and 20 mM Tris-HCl, pH 7.4) containing protease inhibitors (20 μg/ml aprotinin, 2 μg/ml leupeptin, 1 μg/ml pepstatin and 50 μg/ml PMSF). Antigen-antibody complexes from precleared lysates, involving mAb GoH3 and a polyclonal anti-β1 serum, were collected on either Protein A- or G-Sepharose (Pharmacia, Uppsala, Sweden). After washing in lysis buffer of decreasing detergent concentration, agarose beads were finally resuspended in Laemmli loading buffer and incubated 5 minutes at 95°C; in cases of reduction, 5% 2-mercaptoethanol were added. After separation of proteins by 6-8% SDS-PAGE, the gel was dried and exposed to Hyperfilm-MP (Amersham).

Oligonucleotide probes

Probe selection for in situ hybridization was done using the OLIGO™ software (National Biosciences, Inc., Plymouth, MN). Oligonucleotides were purchased from Scandinavian Gene Synthesis AB (Köping, Sweden). To detect integrin α6 mRNA we used a 42mer oligonucleotide probe: 5′-TTC CGG ATC ACG TTG TCC TCG CGG GTG TCC AGG TTG AAG GCT-3′, complementary to nucleotides 247-288 of the mouse integrin α6 mRNA (Hierck et al., 1993).

Integrin β4 was detected using a 45mer oligonucleotide probe: 5′-CCA TCC GGA GGC CAT CAG AGA AGG AGG GTT TCA GGT GGA TGT TGC-3′, complementary to nucleotides 1,588-1,632 of the mouse integrin β4 mRNA (Kennel et al., 1993).

In situ hybridization

In situ hybridization was performed as described using radioactively labelled synthetic oligonucleotides (Durbeej et al., 1993). Embryonic mouse kidneys (day 13 to postnatal day 2) were embedded in TissueTek (Miles Inc., Naperville, IL), frozen in liquid nitrogen, cut (10 μm) on a cryostat and collected on poly-L-lysine coated, sterile microscope slides. Sections were fixed in 4% paraformaldehyde, washed in PBS and dehydrated in ethanol followed by a 5 minute incubation in chloroform. Oligonucleotide probes were 3′ end labelled with [α-^{35}S]dATP (Amersham Sweden AB, Solna) using terminal deoxyribonucleotidyl transferase (Scandinavian Diagnostic Services, Falkenberg, Sweden) to a specific activity of 1×10^9 cpm/μg. Hybridization was performed in 50% formamide, 4× SSC (salt sodium citrate), 1× Denhardt's solution, 10% dextran sulfate, 0.25 mg/ml yeast tRNA, 0.5 mg/ml sheared salmon sperm DNA, 1% sarcosyl, 0.02 M Na_2HPO_4 (pH 7.0) and 0.05 M dithiothreitol. The activity in the hybridization cocktail was 10^7 cpm/ml. Sections were hybridized 15-18 hours at 42°C in a humidified chamber with 0.1 ml hybridization solution per slide. Slides were washed 4× 15 minutes in 1× SSC at 60-65°C. Specific hybridization was determined by using controls where the same amount of labelled probe and an excess of unlabelled probe was allowed to hybridize with sections. Slides were dipped in Kodak NTB-2 photo emulsion, diluted 1:1 in water, exposed 4-6 weeks at 4°C, developed and counterstained with cresyl violet. Photographs were taken, under bright- and dark field illumination, on Kodak T-max 400 film.

RT-PCR

Embryonic (day 14-16), newborn and adult mouse kidneys were collected and frozen at −70°C. Total RNA was extracted (Chirgwin et al., 1979) from pooled samples. Isolation of poly(A) RNA from total RNA was done by affinity chromatography on oligo(dT)-cellulose columns. Reverse transcription (RT) of RNA was done with oligo(dT)-primers (Perkin Elmer, Norwalk, CO) using 1 μg of total RNA. After completion of cDNA-synthesis, enzyme was heat inacti-

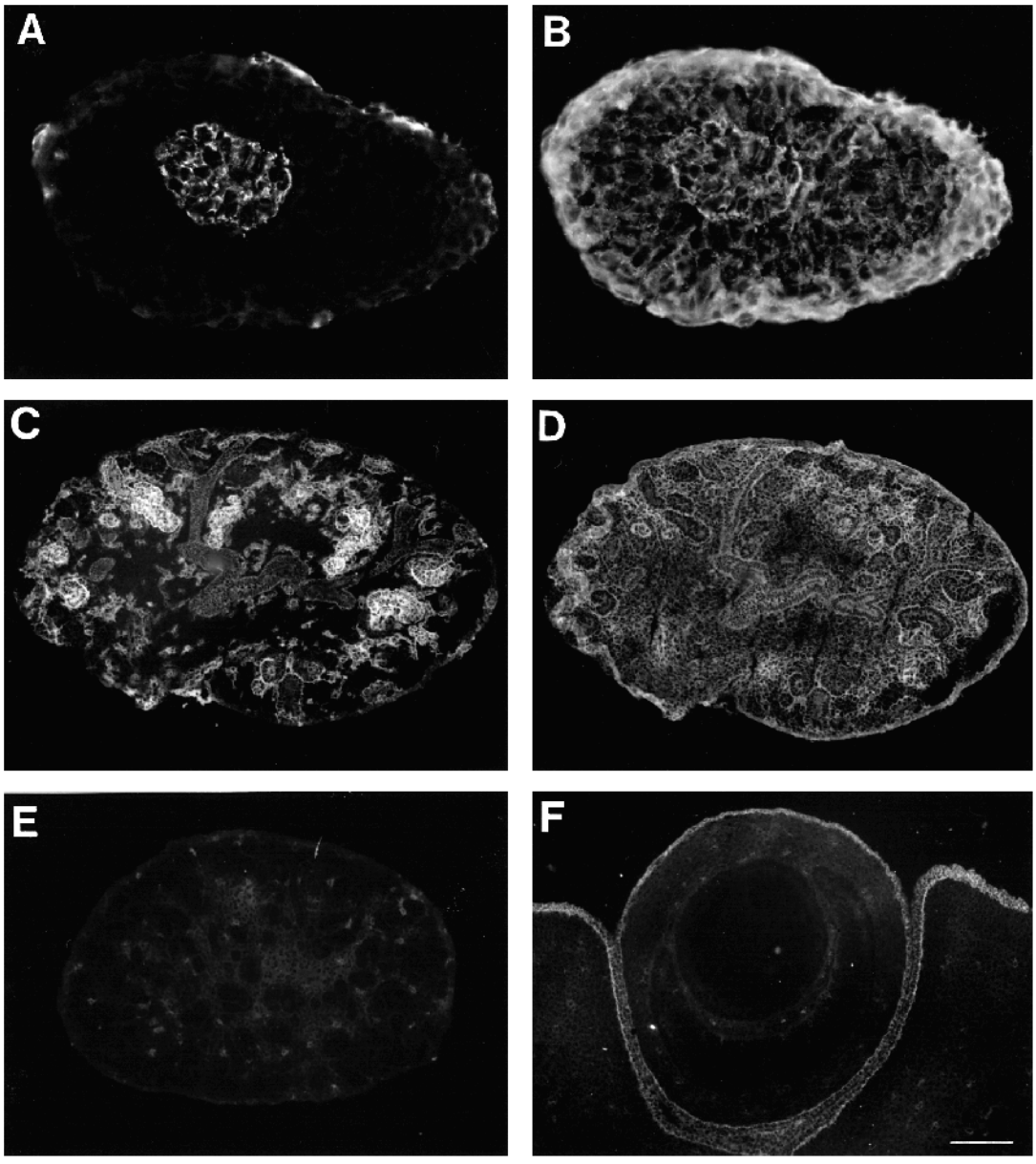

Fig. 1. Immunofluorescence detection of integrin α6, β1 and β4 subunits in embryonic mouse kidney. Double immunofluorescence staining was performed with antibody GoH3 against integrin α6 subunit (A,C) and polyclonal antiserum against integrin β1 subunit (B,D) on sections of kidneys from 11-day-old (A,B) and 14-day-old mice (C,D). Note strong expression of α6 integrin subunit in kidney tubules. Integrin β4 subunit expression was studied by immunofluorescence with antibody SP 90 395 in sections from kidneys (E) and eye region (F) of 14-day embryos. Whereas no staining for β4 integrin subunit was detected in kidney epithelium or mesenchyme from 14-day embryos, faint staining is seen in endothelium (E). In contrast, staining for β4 integrin subunit was seen in epidermis and cornea (F). Bar, 100 μm (A,B) and 200 μm (C,D,E,F).

vated for 5 minutes at 99°C. The PCR reaction was carried out using sense primer 5′-GTCAGGTGTGTGAACATCAGG-3′ (2,975-2,995) and antisense primer 5′-CTGGAAAAAATAAGGGGGGGC-3′ (3,627-3,647) recognizing mouse integrin α6 subunit. Primers were constructed to pick up both the A and B cytoplasmic splice variants. Primers for β1 were sense 5′-AATGTTTCAGTGCAGAGCC-3′ and antisense 5′-TTGGGATGATGTCGGGAC-3′ (Tarone et al., 1993) giving an amplified RT-PCR product of 260 bp. Primers detecting β4 were sense 5′-TAGGGATGTTGAGCCGATGCA-3′ and antisense 5′-CCACTCTCTGACACGCACAG-3′ giving a 259 bp fragment from splice site B (Kennel et al., 1993). Cycles were: 1 minutes denaturation at 94°C, 2 minutes annealing at 55°C and 2 minutes extension at 72°C. PCR products were analyzed on a 1% agarose gel containing ethidium bromide. Products were cloned into the PCR II vector (Invitrogen, CA, USA) and sequenced from both directions. Sequences were run against the GenBank database.

Northern blot analysis

Total RNA was isolated from kidneys or mesenchyme explants frozen in liquid nitrogen (Chirgwin et al., 1979). RNA (10 μg) denatured with glyoxal, was electrophoresed on a 1% agarose gel, transferred to a Zeta-Probe GT membrane (Bio-Rad) and RNA was fixed by UV cross-linking with a Stratalinker (Stratagene). Membranes were prehybridized in 0.25 M Na_2HPO_4 and 7% SDS for 30 minutes at 65°C. Hybridization with cDNAs was performed at 65°C for 18-24 hours in the same solution. Integrin α6 mRNA was detected with a 1.5 kb *Eco*RI cDNA fragment covering positions 2,000-3,490 of the murine α6 subunit (a gift from Dr Arnoud Sonnenberg, Amsterdam). In order to detect β1 integrin mRNA, a 260 bp cDNA fragment covering positions 1,936-2,196 of mouse β1 integrin (Holers et al., 1989) was generated with RT-PCR of mRNA from kidneys from day 14 mouse embryos. A 1.1 kb human G3PDH cDNA probe (Clontech) was used as a control. Probes were labelled with [^{32}P]dCTP to a specific activity of 2×10^8 cpm/μg DNA using an oligolabelling kit (Pharmacia). Filters were washed 2× 1 hour in 20 mM Na_2HPO_4, 5% SDS at 65°C and 2× 1 hour in 20 mM Na_2HPO_4, 1% SDS at the same temperature. Membranes were then exposed to Hyperfilm MP films (Amersham) in the presence of intensifying screens.

RESULTS

Immunofluorescence

In 11-day embryonic mouse kidneys, integrin α6 subunit was found on the surface of ureter epithelium but not in the surrounding mesenchyme (Fig. 1A). The β1 integrin subunit was expressed by all cells of 11-day kidney and particularly well on the basal side of the ureter bud epithelium (Fig. 1B). No β4 integrin subunit could be detected in 11-day kidneys (data not shown). In kidneys from 14-day embryonic mice, α6 integrin subunit could be detected in the developing collecting ducts derived from the ureter bud, but particularly strong expression was noted in newly forming tubules (Fig. 1C). In addition, expression of integrin α6 subunit was noted on the cell surface of blood vessel endothelium. Double immunofluorescence of the sections from 14-day-old mice showed expression of β1 integrin subunit both in mesenchyme, epithelium and endothelium (Fig. 1D). No staining for β4 integrin subunit was found in kidneys from 14-day embryonic mice in any epithelia either in the cortex or medulla (Fig. 1E), although the antibody reacted well with the cell surfaces of skin and corneal epithelium (Fig. 1F). In the embryonic kidney, staining could be seen only in a few blood vessels with the anti-β4 antibody, but the staining was extremely faint (Fig. 1E).

In situ hybridization

In situ hybridization of kidneys from 14-day embryonic mice showed strong expression of integrin α6 subunit mRNA in developing tubules, in epithelium derived from ureter bud and in blood vessels (Fig. 2A,B). No expression of β4 integrin subunit mRNA was found within the kidney either in mesenchyme, epithelium or endothelium (Fig. 2C) although the same oligonucleotide probe reacts strongly with epidermis (Fig. 3B) or embryonic tooth epithelium (Salmivirta et al., 1996).

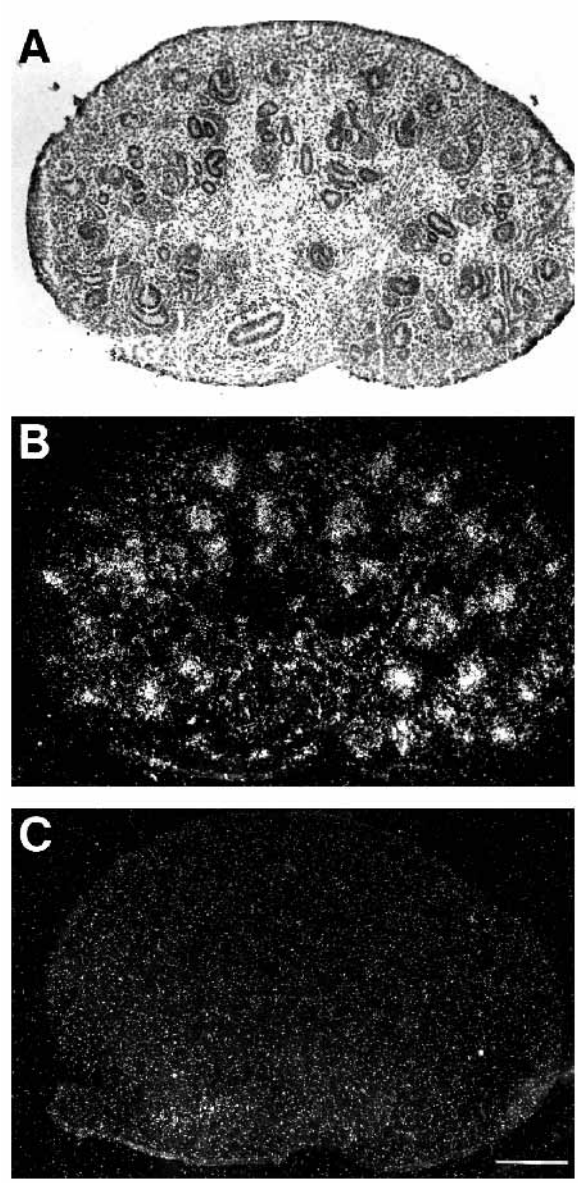

Fig. 2. Detection of mRNA for integrin α6 and β4 subunits in sections of kidneys from 14-day embryos by in situ hybridization. Bright field (A) and dark field (B) micrographs of sections probed with a synthetic oligonucleotide specific for integrin α6 subunit reveal strong expression in all epithelial and endothelial structures. Dark field micrograph of kidney sections probed with the oligonucleotide specific for β4 integrin subunit showed no grains over background in any part of the section (C). Bar, 200 μm.

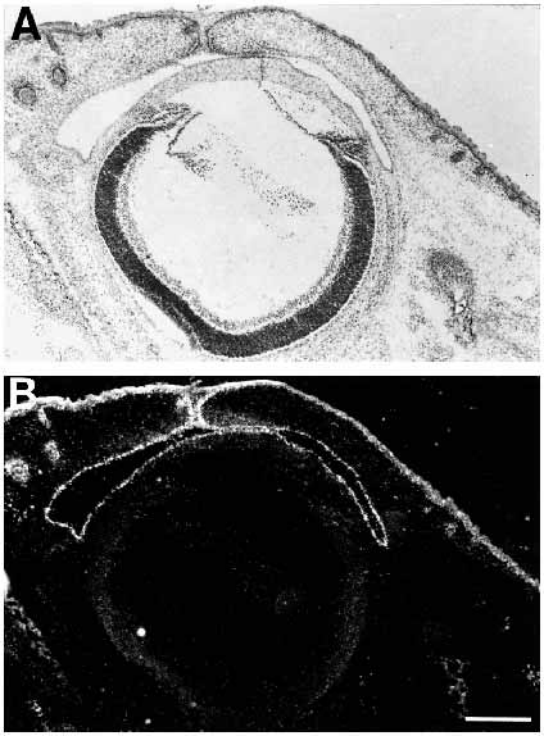

Fig. 3. In situ hybridization on the eye region of a 14-day mouse embryo using an oligonucleotide probe detecting mouse integrin β4 subunit. The section was examined by bright field (A) and dark field microscopy (B). Strong expression is seen in epidermis and cornea (B). Bar, 200 μm.

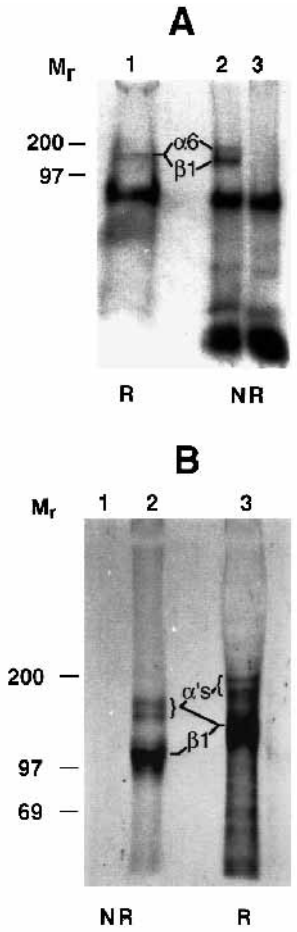

Fig. 4. Immunoprecipitation of metabolically labelled kidneys from 14-day embryos. Antibodies used were either a rat mAb (GoH3) against integrin α6 subunit (A), or rabbit polyclonal antiserum recognizing integrin β1 subunit (B). After immunoprecipitation proteins were separated with SDS-PAGE and run either under reducing (R) or non-reducing conditions (NR). Lanes 3 (A) and 1 (B) are controls with preimmune sera. In A, an unspecific band of about 80 kDa is precipitated. Samples were run on 4-15% (A) or 6% (B) polyacrylamide gels in the presence of SDS.

Immunoprecipitation with antibodies against α6 or β1 integrin subunits

To more directly investigate whether integrin α6 subunit is associated with β1 or β4 subunits, extracts from metabolically labelled embryonic kidneys were immunoprecipitated with chain-specific antibodies. When immunoprecipitations were performed using rat mAb GoH3 against the α6 subunit, only one clear band of 130 kDa was detected under reducing conditions (Fig. 4A, lane 1) This is the expected size of both β1 and α6 integrin subunits under reduced conditions. Reduction of integrin α6 subunit leads to cleavage of a 25 kDa polypeptide, leading to an apparent reduction of molecular mass from 150 kDa to about 130 kDa (Sonnenberg et al., 1990a,b). Under non-reducing conditions, the material precipitated with antibody against α6 integrin subunit was resolved into two bands of 110 kDa and 150 kDa, corresponding to the size of β1 and α6 integrin subunits respectively (Fig. 4A, lane 2). No bands were seen in the 200 kDa region where β4 integrin subunits migrate (Fig. 4A). The strong band of approximately 80 kDa in lane 2 was seen also without the presence of the first antibody (Fig. 4A, lane 3).

Polyclonal antibodies against integrin β1 subunit precipitated polypeptides which under non-reducing conditions had an approximate molecular mass of 110 kDa, 150 and 170 kDa (Fig. 4B, lane 2). The 110 kDa band corresponds to the known size of the β1 integrin subunit, and the 150 kDa to the size of the α6 subunit, whereas the identity of the 170 kDa band is unclear. When precipitates obtained with the anti-β1 integrin antibody were run under reduced conditions, we detected the expected 130 kDa band which could be a mixture of the β1 integrin and the α6 integrin subunits. In addition we detected two bands of approximately 180 and 200 kDa (Fig. 4, lane 3). These bands could be unidentified α chains.

RT-PCR

Two cytoplasmic splice variants of α6 integrin are known, α6A and α6B. In adult kidney only the α6B variant has been detected (Hogervorst et al., 1991; Tamura et al., 1991). In kidneys from 14 or 16 embryos, the 543 bp RT-PCR product representing the B variant was predominant. The 673 bp product representing the A variant was also detected but at much lower levels (Fig. 5). The 673 bp PCR product was identified as the α6A variant also by sequencing. RT-PCR from 14-day kidney RNA using primers amplifying β1 and β4 (Fig. 6) from the same RT-reaction showed a strong 260 bp band representing β1 and a very faint band representing β4 of approx-

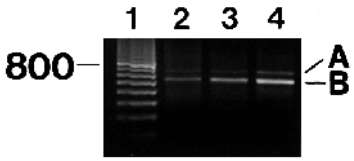

Fig. 5. PCR amplification of reverse transcribed mRNA from 14-day (lane 2) and 16-day (lane 3) embryonic mouse kidneys using primers for two alternative cytoplasmic splice variants α6A and α6B integrin (Hierck et al., 1993). Lane 1 was loaded with molecular markers, and the 800 bp marker is indicated. Markers and PCR products were run on a 1% agarose gel and stained with ethidium bromide.

imately the same size (259 bp). The upper band in the amplification reaction for β4 subunit was also detected when the reverse transcriptase enzyme was omitted, and thus does not represent expression of the β4 subunit (Fig. 6, lane 6).

Northern blot analysis

In kidneys from 11-day-old mice, low expression of the 6 kb message for α6 integrin subunit was detected. Expression was higher at later developmental stages and in kidneys from newborn mice. In the adult stage mRNA expression was lower than in embryonic stages, but the message was still clearly detectable (Fig. 7). To more carefully investigate the relationship between epithelial morphogenesis and expression of α6- and β1 integrin mRNA, different stages of in vitro morphogenesis of kidney mesenchyme were studied. Signals for the α6 subunit were very low in uninduced mesenchyme, and in mesenchyme induced to differentiate in vitro for 24 hours (Fig. 8A,B). At 48 hours a strong expression was noted and the signal intensity remained high at 120 hours of in vitro development (Fig. 8A). Signals for β1 subunit were detected already in the uninduced mesenchyme (Fig. 8A), but expression levels increased to some extent during subsequent stages of conversion of mesenchyme to epithelium (Fig. 8A,C).

Functional assays

Embryonic 12-day mouse kidneys were cultured in the presence or absence of various blocking antibodies. In the absence of antibodies the ureter epithelium branched well and induced the formation of numerous tubules into the mesenchyme (Fig. 9A,B; Table 1). When kidneys were cultured in

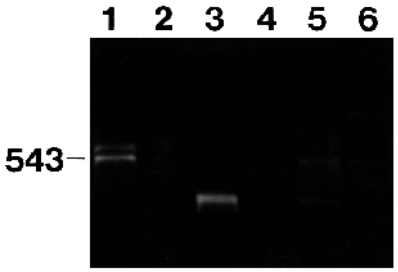

Fig. 6. RT-PCR of 14-day embryonic mouse kidney total RNA using primers for integrin α6 (lane 1), β1 (lane 3) and β4 (lane 5). Lanes 2,4 and 6 are RT controls for α6, β1 and β4, respectively. As a size marker the α6B product of 543 bp is indicated; the size standard is not shown. Products were run on a 1% agarose gel and stained with ethidium bromide.

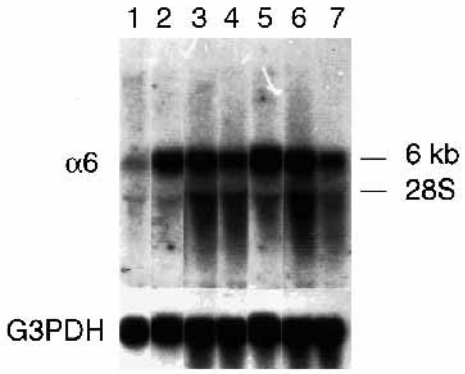

Fig. 7. Time course study of expression of integrin α6 mRNA levels in embryonic and adult mouse kidney as detected by northern blotting. Each lane was loaded with 10 μg of total RNA, blotted and hybridized to a 1.5 kb cDNA probe recognizing the mouse integrin α6 subunit. Stages examined were: lane 1 (day 11), lane 2 (day 14), lane 3 (day 15), lane 4 (day 16), lane 5 (day 18), lane 6 (newborn) and lane 7 (adult). To estimate the amount of total RNA loaded, the filter was also hybridized to a cDNA probe against G3PDH (bottom).

the presence of 100 μg/ml of either antibody GoH3 against integrin α6 subunit (Fig. 9C) or antibody 9EG7 against integrin β1 subunit (Fig. 9D) kidneys were smaller and had fewer ureter branches and tubules. In sharp contrast, kidneys grew well and epithelial sheets differentiated well in the presence of either 100 μg/ml of antibody DECMA against E-cadherin or 100 μg/ml of rat IgG (Table 1).

Histology of the explants showed well formed ureteric and tubular epithelium and a few stromal cells in control cultures without antibodies (Fig. 10A) or 100 μg/ml of rat IgG (Fig. 10B), but in explants treated with either GoH3 or 9EG7 very few if any new tubules could be detected. Instead we detected large areas of dead cells surrounding the ureter epithelium both in GoH3-treated (Fig. 10C) and 9EG7-treated explants (Fig. 10D). It is noteworthy that the ureter epithelium remained intact with polarized cells organized to an epithelial sheet in GoH3-treated explants (Fig. 10C,D).

DISCUSSION

The formation of tube-like structures from less organized stem cells is one of the most fundamental processes during embryogenesis. Tube formation is an essential feature of formation of both blood vessels and epithelia. In the embryonic kidney tubules develop as a result of a conversion of mesenchymal stem cell into epithelial cells. Our previous studies have suggested that cell-matrix interactions are important for kidney tubulogenesis. Although it has been shown that laminin-1 is crucial for kidney tubulogenesis in organ culture (Klein et al., 1988), the subunit composition of the integrin receptors for laminin-1 during kidney tubulogenesis has not been established. With a large number of different assays we now show that the embryonic kidney epithelium contains integrin α6β1 rather than α6β4 as a laminin-1 receptor. We also demonstrate that the α6B variant is the predominant splice variant in the embryonic kidney. Furthermore, blocking antibodies against the α6 and the β1 integrin chains perturbed kidney tubulogenesis in vitro.

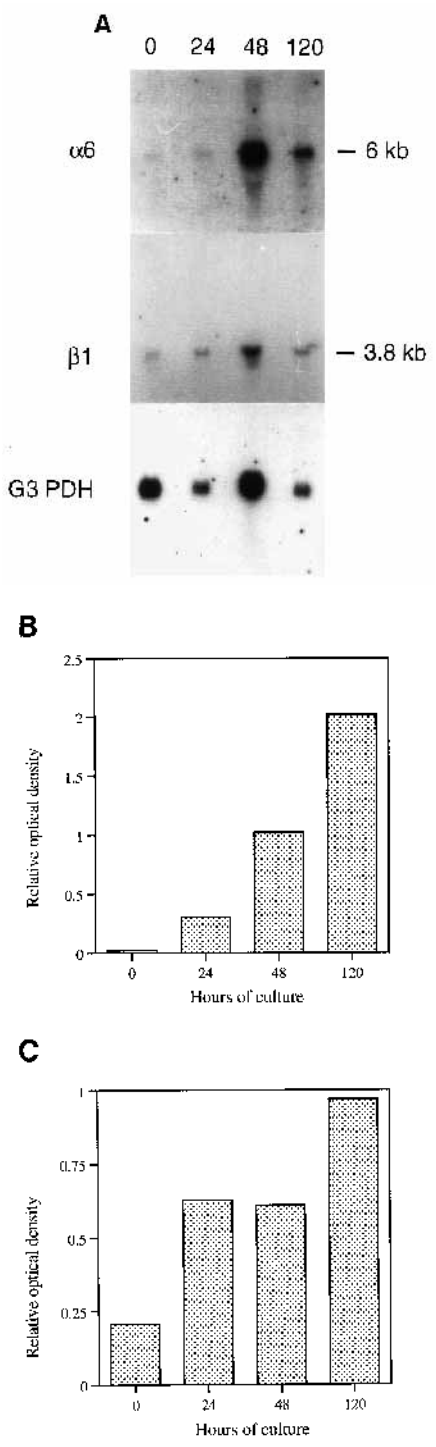

Fig. 8. Integrin α6- and β1 subunit mRNA expression during conversion of metanephrogenic mesenchyme to epithelium in organ culture. Kidneys were dissected from 11-day embryonic mice and the ureter was removed. Isolated mesenchymes were induced to differentiate in vitro by an inducer tissue (spinal cord), giving rise to mesenchymally derived epithelium. Explants were collected at various stages (0-120 hours) and total RNA was isolated. Northern blot analysis was performed using a 1.5 kb integrin α6 subunit cDNA probe, a 260 bp PCR fragment detecting the β1 integrin subunit and a probe for G3PDH (A). (B,C) Histograms show the signal intensities for integrin α6 (B) and β1 subunits (C) normalized for the signal intensities of G3PDH.

Integrin β1 subunit could be easily detected in embryonic kidney by immunofluorescence, as expected from previous studies of human embryonic kidneys (Korhonen et al., 1990a,b). Expression was noted in all cell types, including the developing epithelium. All parts of the nephric epithelium were expressing β1 integrin subunit. In contrast, we failed to detect β4 integrin subunit by immunofluorescence of embryonic kidney epithelium although the same antibody showed integrin β4 expression basally in epidermis. Masking of epitopes can be a problem in immunofluorescence and we therefore analyzed expression of the β4 integrin mRNA in tissue sections. We failed to detect β4 integrin mRNA in embryonic kidneys also by in situ hybridization although we readily detected the message in other locations such as skin (Salmivirta et al., 1996). Previous studies concerning integrin expression in embryonic and adult mouse and human kidney show some discrepancy regarding the localization of integrin β4 subunit. While Natali et al. (1992) reported expression of β4 in human renal tubules with coexpression of both α6 and β1 integrin subunits in the basolateral cell surface, other investigators failed to detect integrin β4 in adult kidney (Sonnenberg et al., 1990a,b; Korhonen et al., 1990b). In a cultured cell line derived from kidney, there seems to be very little α6β1 but instead several other integrins including α6β4 (Schoenenberger et al., 1994). Nevertheless, our studies strongly suggest that the epithelial cells in the embryonic kidney do not express any significant amount of β4 integrin subunit, but rather the β1 integrin subunit.

Immunofluorescence revealed that integrin α6 and β1 subunits colocalize in all kidney epithelial structures from the earliest day examined and throughout in vivo development. The most prominent expression of integrin α6 subunit was seen in developing tubules, whereas integrin β1 subunit was more prominently expressed in ureter bud derived epithelium than in tubules. Integrin α6 subunit was confined to endothelium and epithelium, but integrin β1 subunit was also expressed on mesenchymal cells. The focal expression of α6 integrin subunit by epithelial and endothelial cells could be verified by in situ hybridization. Moreover, northern hybridization matched the localization studies very well. The expected 6 kb message could be detected, and message levels were particularly high during embryonic organogenesis.

Coexpression of both the mRNAs for β1 and α6 subunits was noted also during kidney tubulogenesis in vitro. By isolating the nephrogenic mesenchyme from 11-day embryonic mice and by in vitro culture of the mesenchyme, we could precisely follow expression during onset of epithelial morphogenesis. The mRNA for the β1 subunit could be detected already in uninduced mesenchyme, whereas expression of the α6 subunit was still very low at this stage. During the in vitro conversion of mesenchyme to epithelium, the major increase in the integrin α6 message level occurred on day two of in vitro development, when the first morphological signs of epithelial morphogenesis can be seen. Similar increases in message levels on day two of in vitro development have previously been reported for laminin α1 chain (Ekblom et al., 1990) and the other laminin receptor, dystroglycan (Durbeej et al., 1995).

Although the expression studies were convincing, they could nevertheless only suggest the chain association α6β1. The association of integrin α6 with β1 subunit was verified more directly by immunoprecipitation. The antibodies against β1

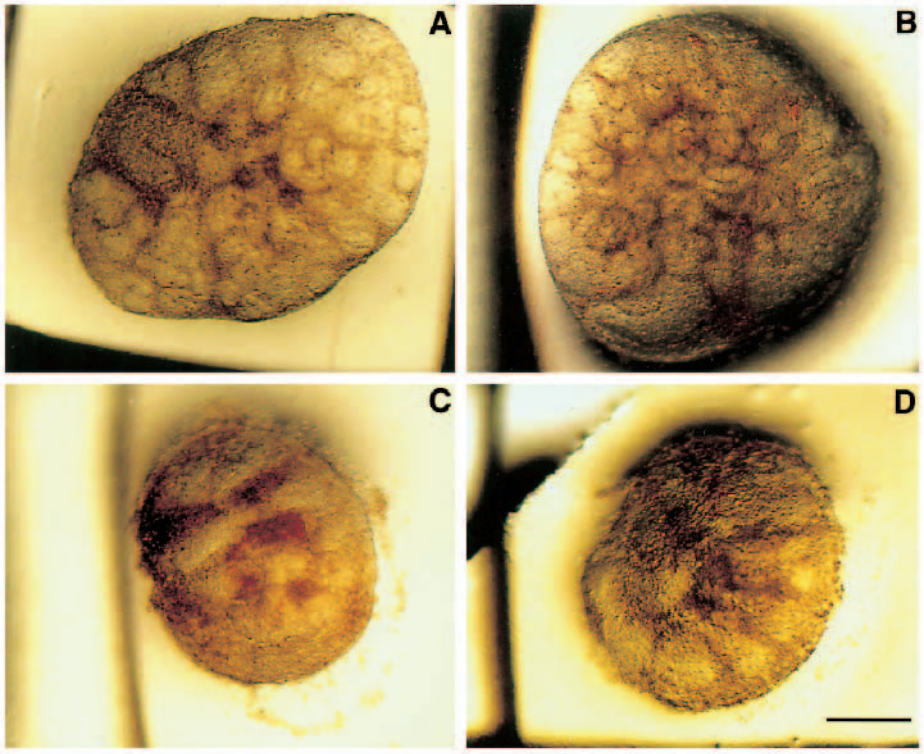

Fig. 9. Perturbation of kidney development with monoclonal antibodies against integrin α6 and β1 subunits. Organ cultures of kidneys from 12-day embryonic mice were photographed by stereomicroscopy on day 3 of in vitro development. Micrographs show kidneys grown in medium without added antibodies (A) in the presence of 100 μg/ml normal rat Ig G (B), in the presence of 100 μg/ml of mAb GoH3 (C) or 100 μg/ml of mAb 9EG7 (D). Bar, 300 μm.

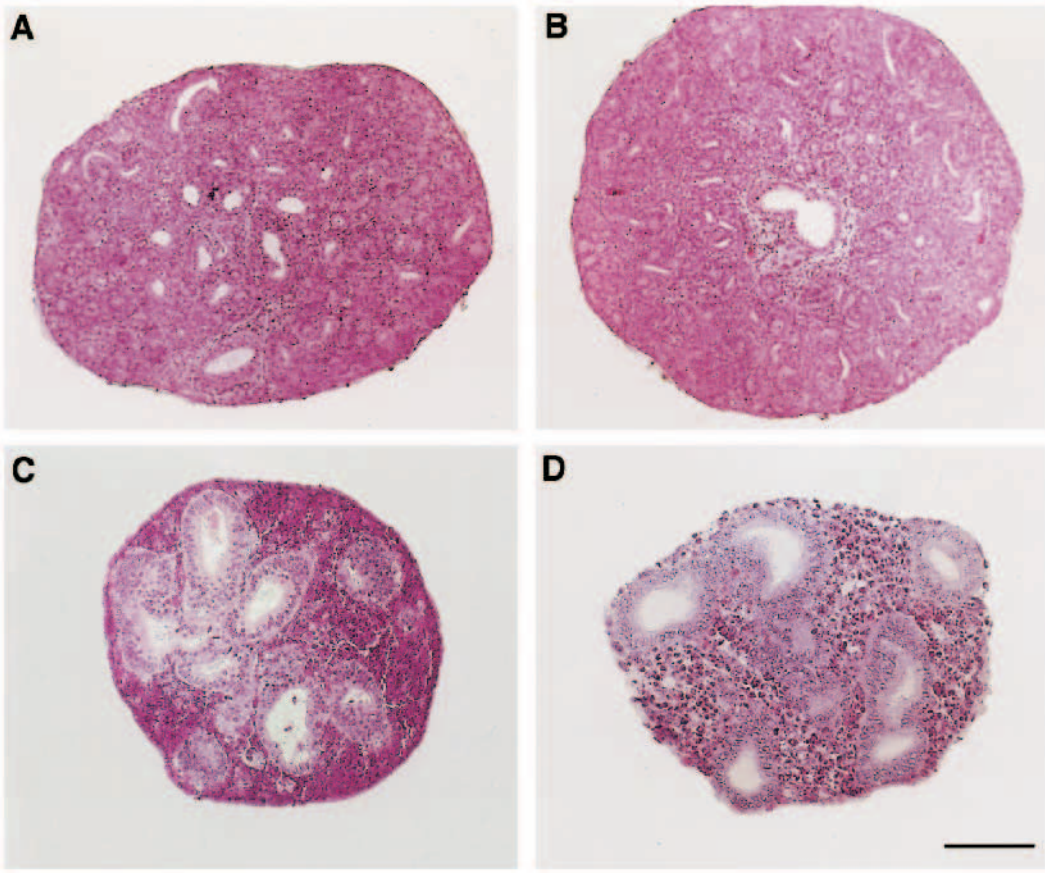

Fig. 10. Histology of embryonic kidney explants cultured in the absence or presence of antibodies against α6 or β1 integrin subunits. Paraffin embedded sections were stained with hematoxylin-eosin. Kidneys from 12-day embryos were cultured in vitro for 3 days in the absence of antibodies (A), presence of IgG (B) or in the presence of monoclonal antibody GoH3 at 100 μg/ml (C) or mAb 9EG7 at 100 μg/ml (D). Note that very few new tubular structures are formed in explants treated with either GoH3 or 9EG7, and all large epithelial cell structures seen are derived from ureter epithelium. In GoH3- and 9EG7-treated explants the area around the epithelial ureter cells is necrotic. Bar, 200 μm (A,B) and 100 μm (C,D).

Table 1. The effect of antibodies recognizing integrins α6 (GoH3), β1 (9EG7) and E-cadherin (DECMA), on tubule formation (mesenchyme condensation), in the kidney in vitro organ culture system

Antibody added	Concn of antibody	Number of kidneys	Number of condensates	Standard deviation
GoH3	100 μg/ml	10	9	2.4
9EG7	150 μg/ml	10	6.6	3.2
9EG7	100 μg/ml	29	15.7	5.5
9EG7	50 μg/ml	18	21.5	8.1
DECMA	100 μg/ml	10	52.6	12.5
No antibody	–	56	47.2	12.1
Rat Ig G	100 μg/ml	31	41.7	14.3

integrin subunit brought down several α subunits in addition to β1. A band in the region expected of the integrin α6 subunit could be detected. Due to similarities in electrophoretic mobility of many integrin α chains, the α6 chain could not be distinguished with certainty from possible other coprecipitated integrin α subunits. When instead immunoprecipitation was performed with GoH3 against integrin α6 subunit, bands corresponding to the size of integrin subunits α6 and β1 were observed. Strong bands migrating in SDS-PAGE in the range expected of β1 integrin subunits could be detected both under reduced and unreduced conditions, whereas no bands representing β4 subunits were seen.

Splice variants with different intracellular domains of the integrin α6 subunit have been reported, and interesting developmentally regulated switches in the expression patterns of these two variants have been reported (Tamura et al., 1991; Hogervorst et al., 1993; Hierck et al., 1993). Our current RT-PCR analyses of the embryonic kidney revealed a predominance of the α6B variant at all developmental stages analyzed. Taken together the results clearly show that embryonic kidney tubules express integrin α6β1 rather than α6β4, and that the α6Bβ1 variant is the predominant form. This is of interest since there now is some evidence that the two α6 splice variants might have distinct functions; the α6Aβ1 integrin has been shown to be more effective than α6Bβ1 in inducing tyrosine phosphorylation of three intracellular proteins (Shaw et al., 1995). These phosphorylation events are thus apparently not important for kidney tubulogenesis.

Finally, we studied the role of integrin subunits α6 and β1 and E-cadherin, using available blocking antibodies (Vestweber and Kemler, 1985; Sonnenberg et al., 1990a,b; Lenter et al., 1993). In previous studies we had already shown that antibodies against α6 integrin subunit can block kidney tubulogenesis in transfilter cultures of isolated mesenchyme (Sorokin et al., 1990). In the current study we used a technically easier assay where both the ureter epithelium and mesenchyme are cocultured, and we could study both ureter development and tubule development. In the currently used assay, antibodies against both the integrin subunits α6 and β1 perturbed kidney development. In sharp contrast, antibodies against E-cadherin in no way seemed to alter development. Lack of effect of E-cadherin antibodies on kidney development was noted already some time ago in experiments in the transfilter assay (Vestweber et al., 1985), but we nevertheless wished to test whether some effects could be seen in another assay. The results leave open the question of whether E-cadherin is required for kidney tubulogenesis. One possibility is that several cadherins participate in kidney tubulogenesis. Kidney-specific cadherins have been reported, and during kidney development many cadherins are expressed by the developing epithelium (Hatta et al., 1987; Xiang et al., 1994).

It will now be an interesting task to study how the antibodies against integrin α6Bβ1 affect in vitro epithelial morphogenesis at the molecular level. The antibodies might act by disrupting cell attachment to laminin-1, but they may also cause an abnormal signaling by the integrin. A further possibility is that the antibodies by steric hindrance affect other cell surface components such as dystroglycan (Durbeej et al., 1995). The histology of the antibody-treated explants suggest that developing tubules die by a three-day long treatment of antibodies either against α6 or β1 integrin subunit. It was noteworthy that the ureter epithelium in contrast to the tubules survived the treatment with antibodies against α6 or β1 integrin subunit rather well. Disruption of cell-matrix interactions may cause apoptosis (Meredith et al., 1993; Frisch and Francis, 1994). One possibility is that the application of antibodies against integrin α6B subunit or the β1 subunit increase apoptosis in developing kidney tubules.

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