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Galectin-3 expression and effects on cyst enlargement and tubulogenesis in kidney epithelial MDCK cells cultured in three-dimensional matrices in vitro

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SUMMARY

Galectin-3 is a member of a closely related family of β-galactoside-binding soluble proteins found in many vertebrate epithelial and myeloid cell types. The developmentally regulated presence of galectin-3 in tissues, for example kidney, and an affinity for many cell-surface and matrix glycoproteins indicate its importance in extracellular biological processes. Since a polarised expression and secretion of galectin-3 was observed in monolayer-cultured MDCK cells, an understanding of the secretion and distribution of this lectin in a three-dimensional in vitro model would help to uncover its role(s) in the interplay between cell-surface adhesion molecules and extracellular matrix components occurring during cell aggregation and polarisation in tissue formation. In this study, the cellular distribution and secretion of galectin-3 were examined in MDCK cells cultured within a gel matrix. MDCK cells were cultured within type I collagen or Matrigel to obtain multicellular cysts, and tubule formation was induced in collagen gels with hepatocyte growth factor. Immunofluorescent staining of these structures using antibodies against galectin-3 and other cell-surface domain markers was carried out either in situ or on cryosections and was visualised by confocal and conventional epifluorescence microscopy. Our results show that MDCK cells suspended in hydrated collagen gels or Matrigel exhibit differential and polarised galectin-3 expression on the baso-lateral surface domains of cells lining the cysts. The lectin is co-localised with laminin on the basal surface. In tubule-forming cysts, galectin-3 is excluded from the initial spikes and the progressing tips of the tubules although its baso-lateral expression on the cyst body remains. Galectin-3 added exogenously to cultures, as well as antibodies against laminin subunits and integrin β_1 subunit, exerted an inhibitory effect on cyst enlargement of MDCK cells in 3-D Matrigel while galectin-3-specific antibodies could promote this process. The results suggest that galectin-3 exerts its effect on MDCK cells in a three-dimensional environment through modulation of both cell-cell and cell-substratum adhesions, and the interplay between these adhesions is important in the growth of multicellular aggregates and extensions occurring during normal kidney tubulogenesis.

Key words: galectin, MDCK cell, 3-D culture

INTRODUCTION

Galectin-3 is a member of a family of β-galactoside-binding mammalian lectins with related amino acid sequences. The lectin, isolated from a variety of epithelial tissues as a monomer with a relative molecular mass (M_r) of approximately 30×10^3, consists of a short N-terminal domain, a proline- and glycine-rich domain of nine amino acid residue repeats and a C-terminal carbohydrate-binding domain (Barondes et al., 1994). Galectin-3, like other members of the family, does not possess a signal peptide or a transmembrane domain, although it is secreted to greater or lesser extent by all cell types examined via an atypical secretory mechanism, similar to that shown by interleukin-1β and bFGF, specifically through the apical surface of polarised MDCK epithelial cells grown on permeable filters (Sato et al., 1993; Lindstedt et al., 1993). Calcium ionophore A23187, valinomycin and stress such as inflammation or heat shock significantly potentiate the secretion of the lectin (Sato et al., 1993).

The precise function(s) of galectin-3 remains undetermined. Its high binding affinity for polylactosamine glycans and ABH blood group epitopes (Sato and Hughes, 1992) would tend to suggest certain roles at the epithelial cell surface where such carbohydrates reside, and as receptors for some naturally occurring ECM ligands like laminin that contain many polylactosamine chains. Although galectin-3 appears to be normally monomeric, at high concentrations it self-associates to form multivalent oligomers which could promote cell-cell and cell-substratum interactions (Hsu et al., 1992; Ochieng et al., 1993; Massa et al., 1993; Mehul et al., 1994). Cross-linking by tissue type transglutaminase may also promote such functions in galectin-3 (Mehul et al., 1995). Recent results indicate that galectin-3 can mediate cell-cell adhesion (Woynarowska et al., 1994; Lotan et al., 1994; Inohara and Raz, 1994). In vitro galectin-3 inhibits in a concentration-dependent manner adhesion of BHK cells on a laminin substratum (Sato and Hughes, 1992), possibly by interference with integrin-mediated interactions (Hughes, 1992, 1994). Regarding roles

in affecting cell adhesion to ECM molecules, opposing functions have been assigned to galectin-1, a 14×10^3 M_r protein homologous to the C-terminal carbohydrate-binding domain of galectin-3 (Milos and Zalik, 1983; Cooper et al., 1991; Zhou and Cummings, 1993; Gu et al., 1994; Mahanthappa et al., 1994). Addition of galectin-1 to CHO and F9 cells expressing polylactosaminated surface glycoproteins promoted their attachment to immobilised laminin (Zhou and Cummings, 1993). Similarly, galectin-1 can promote both cell-cell and cell-matrix adhesion of primary rat olfactory neurons cultured on laminin substrata (Mahanthappa et al., 1994). By contrast, galectin-1 inhibits adhesion and spreading of myoblasts on laminin (Cooper et al., 1991) through binding to integrin receptors (Gu et al., 1994). The galectins are not unique in having dual-functional roles as adhesion modulators, similar properties are shown for other ECM proteins like tenascin, thrombospondin and SPARC (osteonectin) (Chiquet-Ehrismann, 1991; Lane and Sage, 1994).

Previously, we showed that galectin-3 expression is very high in kidney tissue during embryonic development and postnatally in the medullary phase of tubule expansion (Foddy et al., 1990). Although the detailed mechanism for the pattern of kidney epithelial differentiation is unknown at present, both soluble and insoluble factors found locally, such as growth factors and cytokines, and cell-adhesion and matrix molecules, have been implicated to play important roles in this process (Taub et al., 1990; Humes and Cieslinsky, 1992; Sorokin et al., 1992; Santos and Nigam, 1993). The Madin-Darby canine kidney (MDCK) cell is a well-studied polarised epithelial cell type which can form polarised cyst structures when cultured within type I collagen gel and, more importantly, can model some of the morphological events of normal kidney development such as formation of branching tubular and ductal structures when grown within type I collagen gels in the presence of fibroblast-conditioned medium or hepatocyte growth factor (HGF) (Montesano et al., 1991a,b). This study started with the idea that galectin-3 is a good candidate for modulating cellular interactions during kidney development, which in turn could alter vital metabolic activities essential for cyst enlargement, such as cell proliferation and fluid accumulation. We have found by immunofluorescence confocal imaging that, in contrast to previous findings with filter-grown MDCK monolayer cultures (Sato et al., 1993), galectin-3 is localised exclusively at the baso-lateral domains of MDCK cells lining the lumen of cysts and tubules formed in hydrated collagen gels. Furthermore, this lectin is absent on the outgrowing tips of most tubular structures formed by MDCK cells under the influence of HGF. In Matrigel cultures in which MDCK cells form cysts but cannot be induced to form tubules by HGF, addition of exogenous galectin-3 significantly decreased the rate of MDCK cyst enlargement. By contrast, polyclonal galectin-3-specific antibodies accelerated cyst expansion.

MATERIALS AND METHODS

Cell lines and reagents

Chemicals and reagents were obtained from Sigma Chemical (Poole, England) unless otherwise specified. MDCK type II cells (Porton Down, Salisbury, England) were grown routinely in monolayer culture at 37°C with 5% CO_2 in 1:1 (v/v) mixture of Dulbecco's Modified Eagle's Medium (DMEM) and Nutrient Mixture Ham's F-12 (Gibco BRL, Paisley, UK) supplemented with 10% (v/v) foetal calf serum, 2 mM L-glutamine, penicillin (100 units/ml) and streptomycin (100 μg/ml). For serum-free culture, DMEM/F-12 (1:1, v/v) mixture was used containing EGF (10 ng/ml), insulin (5 μg/ml), transferrin (5 μg/ml), hydrocortisone (5 μg/ml), bovine serum albumin (20 μg/ml), L-glutamine and antibiotics. Collagen type I from calf skin was from Sigma. Human foetal lung fibroblast MRC 5 cell line from Porton Down was grown routinely in monolayer culture in supplemented DMEM/F-12 as above. Basement membrane Matrigel extracted from the Engelbreth-Holm-Swarm (EHS) mouse sarcoma and human hepatocyte growth factor (HGF) were obtained from Universal Biologicals (London, England). Recombinant hamster galectin-3 was prepared as described (Mehul et al., 1994). *Datura stramonium* agglutinin was from Sigma. Transwell-COL culture insert with a transparent, collagen-treated microporous polycarbonate membrane (12 mm diameter, 0.4 μm pore size) was supplied by Costar (High Wycombe, England). Rabbit polyclonal antisera were raised against hamster galectin-3 and the C-terminal carbohydrate-binding domain (Foddy et al., 1990; Mehul et al., 1994). These antibodies cross-react with canine galectin-3 as shown previously (Sato et al., 1993). Laminin purified from EHS tumour was used to obtain polyclonal antisera in rabbits, which recognised α, β and γ subunits of EHS laminin (Sato and Hughes, 1992). Rabbit polyclonal antisera against the integrin β_1 subunit was provided by Staffan Johansson (Uppsala University, Sweden). Rat monoclonal antibody (IgG2a) against mouse galectin-3 was from Boehringer-Mannheim (Lewes, England). Mouse monoclonal antibody against apical cell surface marker gp135 (Ojakian and Schwimmer, 1988) was kindly provided by Dr I. Burdett (NIMR, London). Fluorescein isothiocyanate (FITC)-conjugated goat anti-rabbit IgG was obtained from ICN Biochemicals (High Wycombe, England). FITC-conjugated goat anti-rat and anti-mouse IgGs were supplied by Sigma.

3-D hydrated collagen gel culture of MDCK cells

Type I collagen from calf skin was dissolved in 0.1% (v/v) glacial acetic acid to a concentration of 3.0 mg/ml; 8 volumes of ice-cold collagen solution was mixed with 1 volume of chilled 10× DMEM medium. The pH of the solution was brought to 7.4±0.2 as monitored using phenol red with 1 volume of diluted NaOH (usually 0.1-0.2 M). MDCK cells were trypsinised from monolayer cultures and added into the ice-chilled neutralised isotonic collagen solution to yield a final concentration of 1×10^5 cells/ml; 1 ml well of cell-containing collagen solution was dispensed into 24-well culture plates, which were then placed in a CO_2-free incubator at 37°C for a minimum of 45 minutes to ensure complete and uniform gelation. The solidified gels were covered with culture medium and incubated at 37°C with 5% CO_2 for up to 2 months. In order to induce tubulogenesis from MDCK cysts formed within gelled collagen, three approaches were employed: addition of human HGF (20 ng/ml) to the culture medium, co-culture with human fibroblast MRC 5 cell line or addition of MRC 5-conditioned medium (50%, v/v) to the growth medium. Co-culture was performed either using a sandwich model in which a top MDCK-laden collagen layer was separated from the bottom MRC 5-containing collagen layer by a blank collagen layer or in a Transwell-COL unit where the top MDCK-containing collagen gel was cultured with a monolayer of MRC 5 cells in the lower chamber.

3-D Matrigel culture of MDCK cells

The commercial Matrigel, usually supplied with concentrations between 12 and 15 mg/ml, was diluted with ice-cold serum-free medium in a ratio of 1:2. Cells were introduced into the cooled Matrigel solution at a concentration of 5×10^4 cells/ml. The Matrigel solution was gently mixed and dispensed into multiwell plates at 0.5 ml/well. The mixture was incubated at 37°C until gelation occurred (up to 30 minutes) and then covered in medium. In some experiments,

the medium was supplemented with lectins (30 μg/ml) or antibodies of various dilutions or a combination of both reagents. The medium containing these reagents was changed every 2 days.

Immunofluorescent staining of MDCK cells

For cells cultured within collagen gels, immunofluorescent staining was performed with the cells in situ. Collagen gel was excised from culture wells using a scalpel and washed on a shaker three times in cold PBS for 3 minutes each. The collagen gel was then fixed in freshly made 4% (w/v) paraformaldehyde in PBS with slight agitation at room temperature for 15 minutes. The fixed collagen gel was rinsed in PBS and then cut into 3 mm^3 pieces. In order to permeabilise the cells to be stained, the diced collagen gels were incubated in 0.5% (v/v) Triton X-100 in PBS at room temperature for 15 minutes. Usually, any excess aldehyde was blocked by incubating gels with 0.5 mg/ml sodium borohydride in PBS for 15 minutes followed by thorough washing in PBS. Nonspecific binding sites on the cells were blocked by 4% (w/v) BSA in PBS. Prebleed normal rabbit serum was used as negative control for polyclonal sera. The primary antibody was appropriately diluted in 1% (w/v) BSA in PBS and applied to the diced gels in sufficient volume to immerse the pieces. Incubation was carried out either at room temperature for 2 hours or at 4°C for 16 hours. The gels were then thoroughly washed in PBS for three times each for 15 minutes. The FITC-conjugated secondary antibodies diluted in 1% BSA-PBS were applied to the collagen gels for 1 hour at room temperature. The gel pieces were then washed three times before being stained with 5 mg/ml propidium iodide in PBS at room temperature for 10 minutes followed by washing in PBS for 10 minutes. The gels were transferred to slides with recesses and mounted in Hydromount medium (National Diagnostics) for either conventional or confocal microscopy. For cells cultured within Matrigel, all primary and secondary antibodies to be used were pre-absorbed on a layer of gelled Matrigel at 37°C for 30 minutes. Because of the fragility of the Matrigel, the culture was aspirated into a Pasteur pipette and the broken Matrigel was removed by low speed centrifugation. The rest of the procedure was the same as for collagen gel cultures. For cryosectioning of the cells in collagen gel or Matrigel, a piece of gel was placed on a drop of OCT embedding medium (Miles), which was quickly immersed in isopropanol/dry ice. The frozen samples were then transferred to pre-cooled freezing vials and stored at −70°C. Sections (10 μm) were cut with a cryostat and stained as described above.

Conventional and confocal fluorescence microscopy

A Zeiss Axiophot fluorescence microscope was used for conventional epifluorescence microscopy and photography. A Bio-Rad MRC 600 laser scanning confocal microscope (LSCM) system with SOM™ and COMOS™ data retrieving and analysing software was used for confocal microscopy. A vertical *z*-axis serial optical sectioning at 1-2 μm intervals with Kalman filter at factor 8 was executed on each stained sample and data were stored on WORM optical discs. For colour imaging of doubly stained samples, pixels collected from both the green and red channel were merged with 256 optimised colours and the resulting images were photographed off a high-resolution monitor screen. None of the image-intensifying facilities in SOM™ or COMOS™ was applied to the final image.

Measurement of cyst diameter in 3-D matrix

The diameters of multicellular cysts formed by MDCK cells in Matrigel was measured both by visual assessment using an eyepiece incorporating a graticule and by on-screen measurement taking advantage of the transmission light recording feature on the MRC-600 confocal system. The culture well was divided into ten equal-sized areas, within each of which the largest cyst was selected across the entire width and depth of the field. The data from the ten fields were averaged and means and standard deviations obtained.

RESULTS

Galectin expression in MDCK cells grown in three-dimensional collagen gel

MDCK cells seeded within a three-dimensional collagen gel showed a sequence of morphological events described fully by others (McAteer et al., 1987; Wang et al., 1990a,b). In brief, clonal growth and formation of small aggregates occurred, which eventually developed into cyst-like structures containing a polarised epithelium surrounding a central lumen (Fig. 1a). The cysts increased in size with further cell divisions during continued culture and often formed a multi-layered epithelium surrounding the lumen. In single cells, galectin was expressed uniformly over the cell surface and within the cytosol (Fig. 2a). As cells multiplied within the 3-D gel and formed loose aggregates, the cytoplasmic staining decreased, often appearing in patches lying underneath the plasma membrane, and predominant surface staining was apparent (Fig. 2b). When cells progressed to form cysts with a single layer of cells lining the lumen, the distribution of galectin-3 became strongly polarised. The lectin was confined to the baso-lateral domains of the cell surface with little cytosolic expression (Fig. 3a) and the intensity of lateral-domain staining tapered off towards the apical (luminal) surface (Fig. 3b). As the cysts enlarged, the cells lining the lumen became multi-layered; the cells directly facing into the lumen retained the baso-lateral distribution of galectin-3 whereas the cells lining the outer periphery of the cyst expressed galectin-3 uniformly over the cell surface (Fig. 4). Cells also expressed variable amounts of cytosolic galectin-3. A strong basal expression of galectin-3 in cells lining the single-layered cysts was also evident in cryostat sections stained with specific antibodies (Fig. 5).

Polarity marker laminin and gp135 in the cysts

Laminin and gp135 (Ojakian and Schwimmer, 1988) were used to mark the basal and apical domains, respectively, of MDCK cysts growing in 3-D collagen gels. Laminin expression was co-localised with galectin-3 at the basal domain (Fig. 6a). The gp135 protein marker, shown previously to be associated with the apical cell surface of polarised MDCK cells (Ojakian and Schwimmer, 1988; Wang et al., 1990a,b), in our hands was found to be expressed on the basal surface as well as the apical domain of MDCK cysts (Fig. 6b). However, clear apical staining was obtained, distinct from the basal distribution of laminin. Some diffuse cytoplasmic staining with gp 135 antibodies was also observed (Fig. 6b).

Galectin-3 expression during tubulogenesis

Tubulogenesis was induced in MDCK cysts grown in 3-D collagen gels by treatment with purified HGF, foetal fibroblast MRC 5-conditioned medium which contains HGF or by co-culture of MDCK cysts with a separate collagen layer containing MRC 5 cells as described by Montesano et al. (1991a,b). Shortly after induction, sprouting of the cysts occurred (Fig. 1b) and these processes developed into multi-cellular arms which branched and eventually formed tubular structures with a central lumen (Fig. 1c and d). The growth and development of the tubules was very similar for the three methods used for induction. Galectin-3 expression in the main

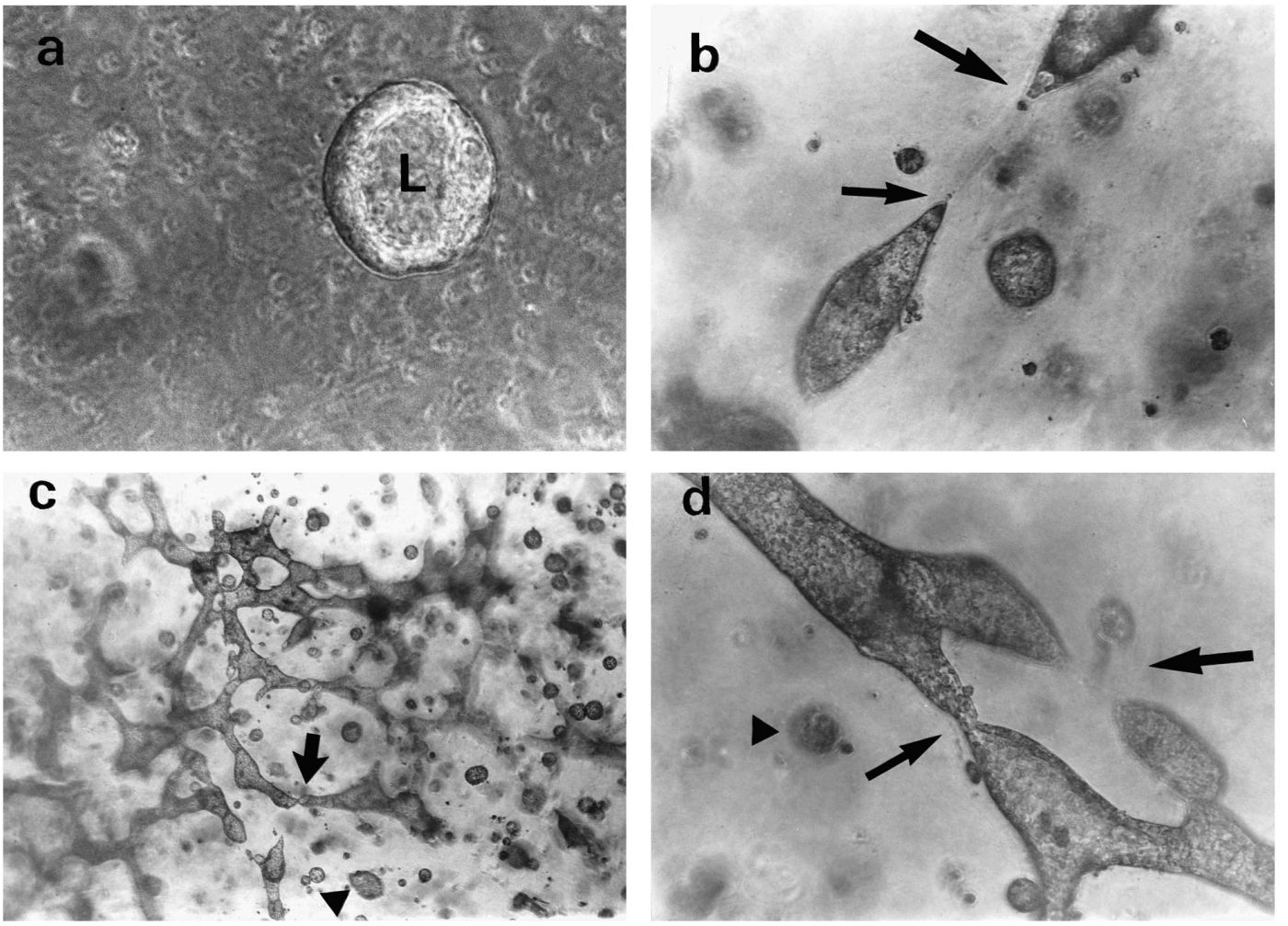

Fig. 1. Morphology of MDCK cells cultured within a collagen gel visualised by phase-contrast microscopy. (a) Appearance after 8 days in culture. The diameter of the cyst is estimated by focusing up and down through the cyst body. A central lumen (L) is clearly visible; (b) after 2 weeks in culture in the presence of HGF (20 ng/ml), the cysts elongate and form sprouts (arrows); (c and d) after 4 weeks in culture with HGF, an extensive network is formed by cyst elongation, branching and fusion. Fusing tubules are indicated (arrows). Note that many spherical cysts (arrowheads) remain even after extended culture. ×400 (a); ×200 (b); ×50 (c); ×200 (d).

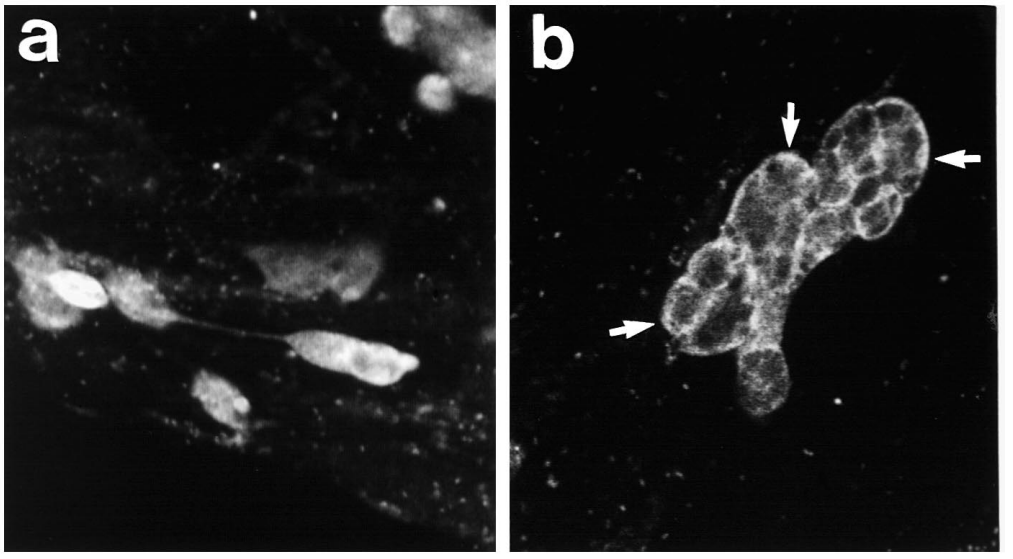

Fig. 2. (a) Immunofluorescent staining of galectin-3 in MDCK single cells suspended within a collagen gel showing mainly diffused cytoplasmic staining; (b) A 4 day old cell aggregate displays galectin-3 mainly at the cell surface. Some focal aggregates of lectin underlying the plasma membrane are also seen (arrows). ×200.

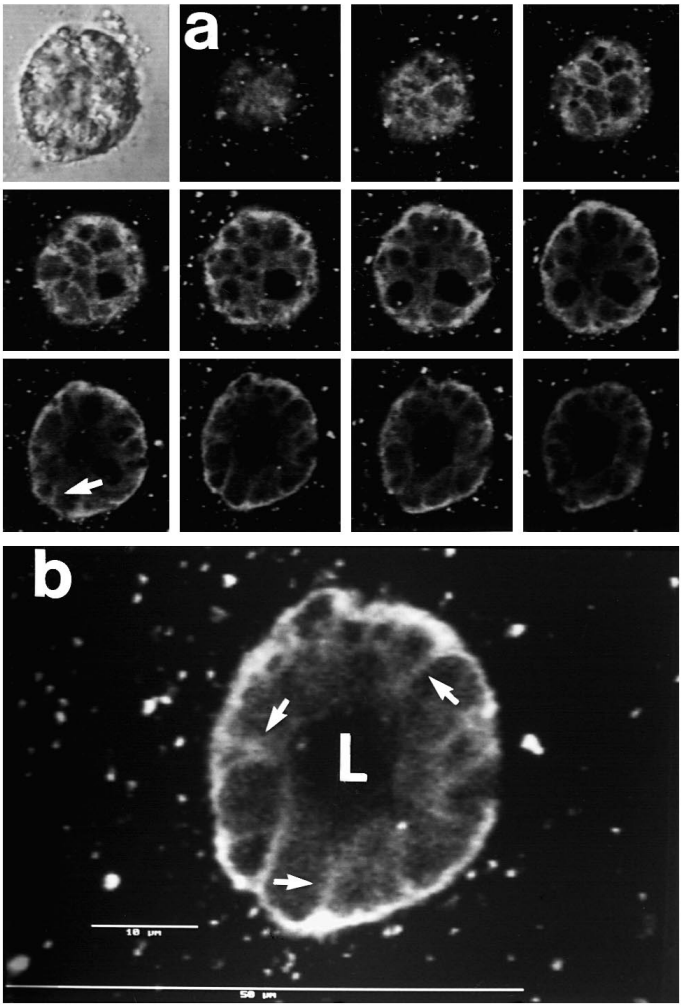

Fig. 3. Galectin-3 staining in 7 day old single-layered MDCK cyst grown in a collagen gel. (a) The cyst is optically sectioned from one pole to the midline at 1 μm intervals. The first of the insets (top left) is a phase-contrast photomicrograph of the cyst. Both the serial sectioning (a) and the enlargement of one section (b) demonstrate that galectin-3 is mainly seen on baso-lateral domains and the level of lectin expression on the lateral domain became progressively reduced (arrows) towards the surface of the lumen (L). ×200.

body of the sprouting cysts appeared similar to that found in simple cysts developing in the absence of HGF: the basal and part of the lateral surfaces were strongly positive (Fig. 7a). Single cells or small numbers (2-3) of cells at discrete sites in the cyst body lacked galectin-3 staining at the cell surface. We presume these to be the sites initiating extension of the multicellular tubules (Fig. 7e). In the latter the tubular regions immediately adjacent to the central cyst body were also stained baso-laterally but the distal portions of the emerging tubules were devoid of galectin-3 (Fig. 7a-c). In the process of fusion of contacting tubules originating from separate cysts, the tubule tips at the point of contact were also free of galectin-3 (Fig. 7e and f). In a multi-sprouting cyst, the tip of each emerging tubular process clearly lacked galectin-3 (Fig. 7d).

Effects of galectin-3 and its antibodies on cyst enlargement in Matrigel

When cultured in Matrigel under serum-free conditions, MDCK cells formed multicellular cysts and enlarged to approximately seven times the original single cell volume in 21 days with the most rapid growth occurring in the third week following a brief plateau (Fig. 8a). In general, we found that cyst enlargement proceeded faster in a three-dimensional Matrigel than in collagen. However, the localisation of galectin-3 during cyst enlargement in Matrigel appeared

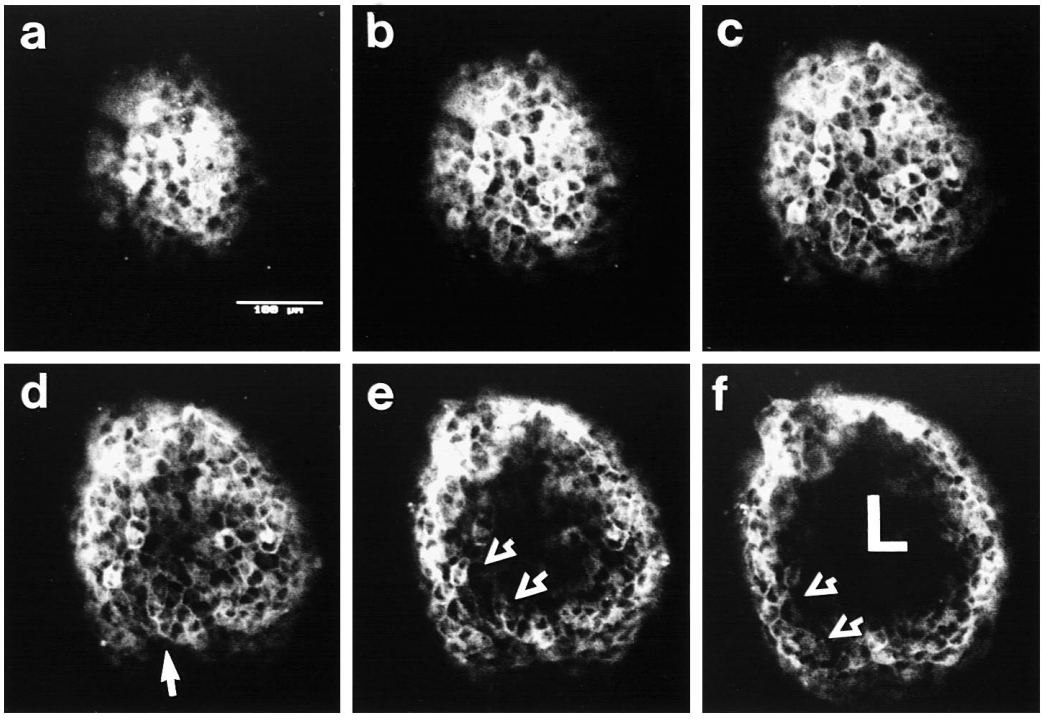

Fig. 4. Optical sectioning of an 8 week old MDCK cyst grown in collagen gel and stained for galectin-3. Sectioning was from the pole (top left) into the lumen (L, bottom right). The cyst is multilayered and galectin-3 is distributed at intercellular spaces (arrows). The free surface of the cells lining the lumen (L) is unstained (arrowheads). ×100. Bar, 100 μm.

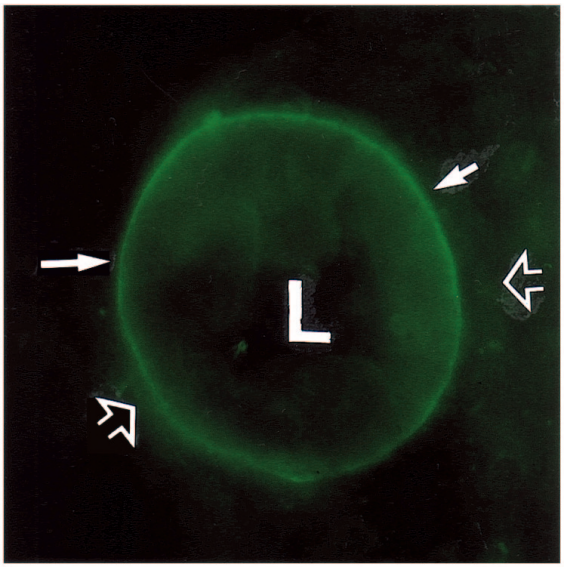

Fig. 5. Epifluoresence photomicrograph of a 10 μm cryosection of a 14 day old MDCK cyst grown in a collagen gel stained with galectin-3-specific antibody. Lectin is distributed mainly at the basal surface of cells lining the cyst (arrows) and between the cells and the surrounding matrix (arrowheads). The lumenal (L) surface is free of galectin-3. ×400.

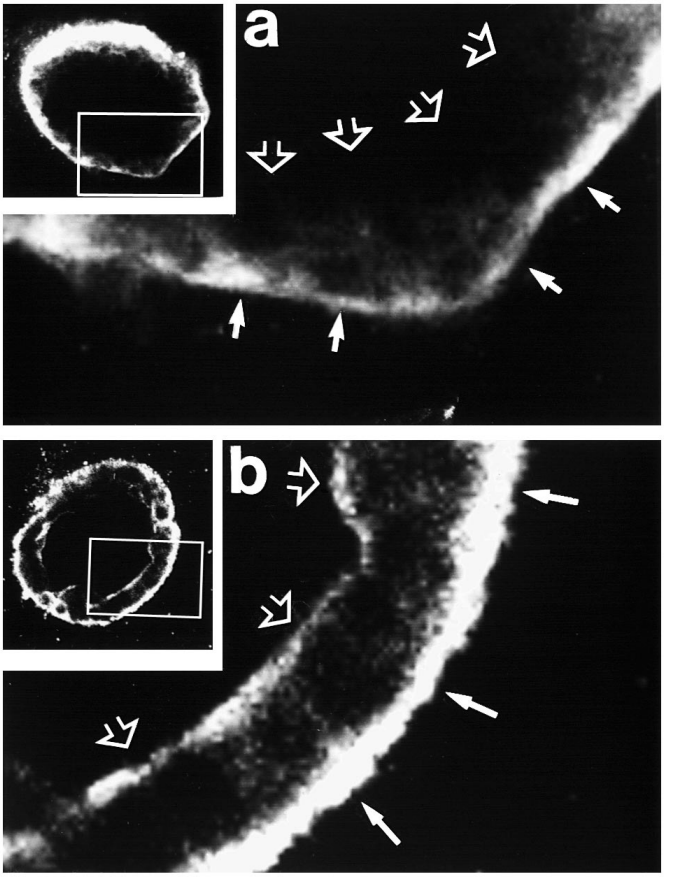

Fig. 6. Immunofluorescence staining of laminin (a) and gp135 (b) in MDCK cells grown as cysts in collagen gels for 14 days. The insets show overall staining of the cyst; magnified enlargements are of the square areas. The basal surface (arrows) and the luminal surface (arrowheads) are indicated. Laminin was predominantly located at the basal surface and gp135, a putative apical marker, was found on basolateral and apical surfaces. ×200 insets.

identical to that found for collagen-grown cysts (results not shown). As described by others (Santos and Nigam, 1993), MDCK cysts grown in Matrigel cannot be induced by HGF to form tubules. Hence, the Matrigel system offers a simple assay to test the effect of exogenous galectin-3 on cyst expansion. When full-length recombinant hamster galectin-3 was applied to the cultures at a concentration of 30 μg/ml, an inhibiting effect on cyst enlargement became apparent after the first 7 days and rapid subsequent enlargement seen in control cultures was largely abolished (Fig. 8a). The inhibiting effect on the rapid phase of enlargement was reversed by removal of galectin-3 from the culture (Fig. 8a) and addition of a neutralising concentration of specific antibody against the carbohydrate-binding domain of galectin-3 (Fig. 8b) or antibody raised against the full lectin (Fig. 8c) which recognises mainly epitopes in the N-terminal domain (Sato et al., 1993; Mehul et al., 1994). Conversely, either of these antibodies when added alone to cultures at 1:25 or 1:50 dilution stimulated cyst enlargement up to 1.5- to 2-fold (Fig. 8b and c). The effects of the antibodies were specific, since neutralisation by addition of galectin-3 abolished their stimulatory action (Fig. 8b and c). Antibodies against laminin subunits and the integrin β_1 subunit also blocked cyst enlargement to an extent similar to that of the addition of exogenous galectin-3 (Fig. 8d). *Datura stramonium* agglutinin, a plant lectin which exhibits similar specificity to that of galectin-3 for polylactosamine [Galβ1-4GlcNAcβ1-3]$_n$ glycans (Yamashita et al., 1987) also inhibited cyst enlargement (Fig. 8d).

DISCUSSION

In this study we present data on the topographical distribution of galectin-3 in MDCK cells differentiating into multicellular cysts during culture within three-dimensional gels consisting of type I collagen or Matrigel, a commercial product made from the extracellular matrix of murine EHS tumour. To our surprise, we found that galectin-3 is selectively localised at the baso-lateral surface of cyst-forming cells in the three-dimensional gels (Fig. 9). This conclusion has been supported by indirect immunofluorescence using confocal microscopy on cell cultures in situ and conventional microscopy of cryostat sections. These results are surprising in as much as previous studies (Sato et al., 1993; Linstedt et al., 1993) clearly showed that galectin-3 is secreted from the apical domain of MDCK cells grown as polarised monolayers on permeable membrane filters and is also present on the apical membranes of such cells.

Present data, many obtained using filter-grown MDCK cells as a model system, strongly support important roles for extracellular signals generated by cell-substratum and cell-cell interactions in the establishment of cell polarity. Such signals may activate additional adhesion molecules, as well as junctional assembly, leading to the formation and stabilisation of a sub-membranous cytoskeleton, which appears to be crucial in re-organisation of organelles within the cell and establishment

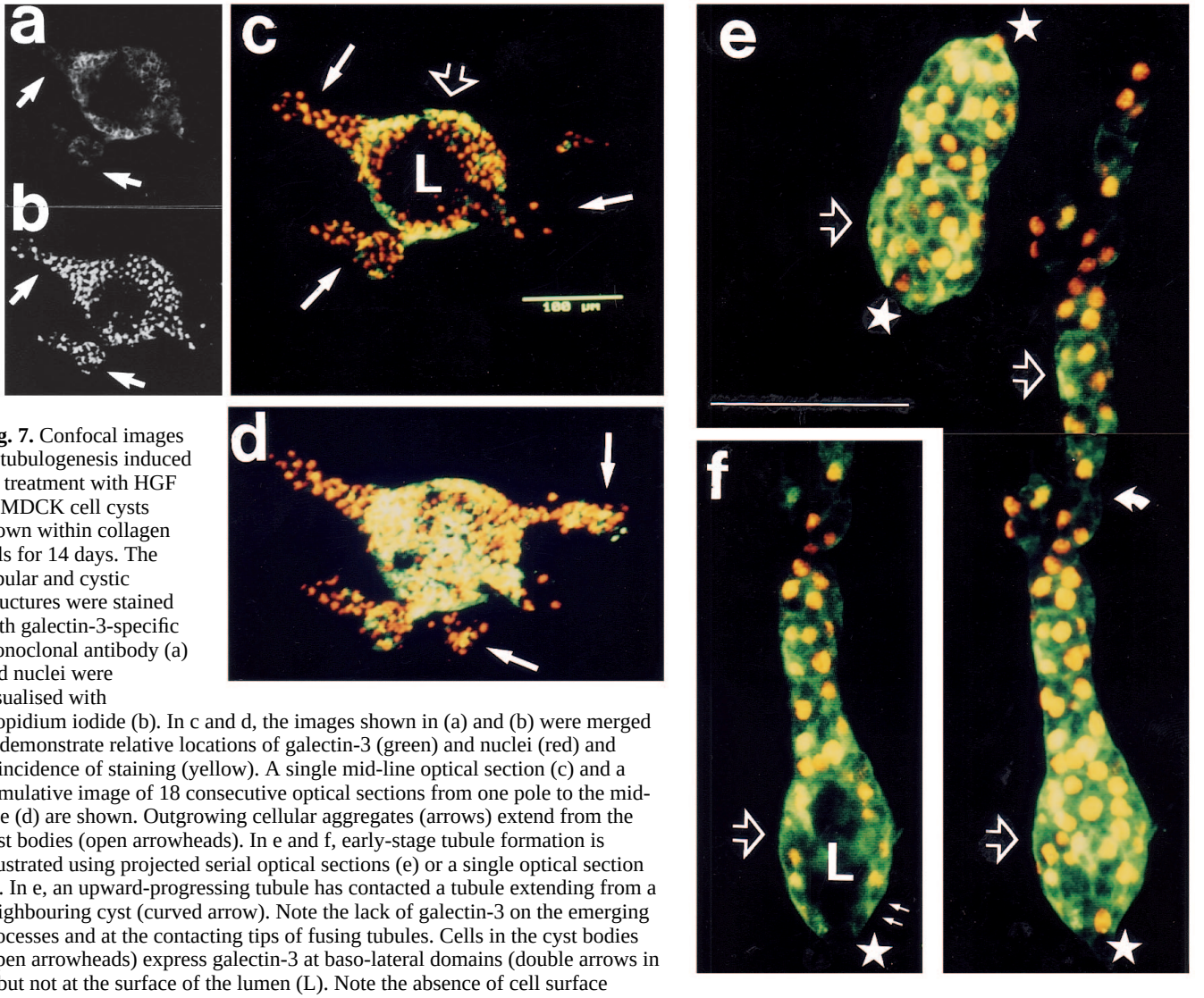

Fig. 7. Confocal images of tubulogenesis induced by treatment with HGF of MDCK cell cysts grown within collagen gels for 14 days. The tubular and cystic structures were stained with galectin-3-specific monoclonal antibody (a) and nuclei were visualised with propidium iodide (b). In c and d, the images shown in (a) and (b) were merged to demonstrate relative locations of galectin-3 (green) and nuclei (red) and coincidence of staining (yellow). A single mid-line optical section (c) and a cumulative image of 18 consecutive optical sections from one pole to the mid-line (d) are shown. Outgrowing cellular aggregates (arrows) extend from the cyst bodies (open arrowheads). In e and f, early-stage tubule formation is illustrated using projected serial optical sections (e) or a single optical section (f). In e, an upward-progressing tubule has contacted a tubule extending from a neighbouring cyst (curved arrow). Note the lack of galectin-3 on the emerging processes and at the contacting tips of fusing tubules. Cells in the cyst bodies (open arrowheads) express galectin-3 at baso-lateral domains (double arrows in f) but not at the surface of the lumen (L). Note the absence of cell surface galectin-3 at discrete single-cell sites (stars) in the cyst body, which presumably are precursors of the multicellular outgrowing aggregates. ×100 (a,b,c and d); ×200 (e and f). Bars, 100 µm.

of pathways whereby membrane and secreted proteins are targeted specifically to apical, lateral or basal domains of the plasma membrane (Simons and Fuller, 1985; Nelson and Hammerton, 1989; Nelson et al., 1990; Nigam and Brenner, 1992). Previous work (Wang et al., 1990a,b) has shown that MDCK cells grown in collagen gels as multicellular aggregates establish a full polarity with formation of tight junctions and a defined epithelial axis similar to that shown by MDCK cells in monolayer culture. However, MDCK cells grown in three-dimensional gels form polarised aggregates in which the apical domain faces into a central enclosed lumen, unlike monolayer cells where the free apical domain contacts the medium. It is possible, but remains to be proven, that these differences in polarised assemblies are sufficient to account for the distinct differences in galectin-3 localisation between polarised cells in monolayer and three-dimensional gel cultures. Other examples are known where the same protein may be targeted either to the apical or to the baso-lateral domain in the one cell type: for example, the Na^+,K^+-ATPase in certain MDCK clones and in thyroid cells (Hammerton et al., 1991; Zurzolo and Rodriguez-Boulan, 1993; Gottari and Caplan, 1993) and band 3 protein in intercalated epithelial cells of renal collecting tubules (van Adelsberg et al., 1994). In the latter case, a specific extracellular matrix protein of M_r 230×10^3 produced by cells at high density was shown to reverse the targeting of band 3 protein from apical to baso-lateral domains, possibly by binding to a baso-lateral receptor to generate signals causing a reorganisation of cytoskeleton towards the baso-lateral membrane and fixation of band 3 protein through interactions with baso-lateral ankyrin. It is possible that synthesis of this or a similar signalling molecule and its incorporation into the matrix laid down by MDCK cysts in three-dimensional gels may induce the retargeting of galectin-3 in the system over a period of observation of several days.

In the new-born hamster kidney, galectin-3 is localised in distal and collecting tubules, where it is expressed only at the apical (luminal) surface (Foddy et al., 1990). The MDCK three-dimensional gel culture system which we have used in the present study morphologically mimics earlier stages of kidney development, i.e. the transition from aggregates of non-

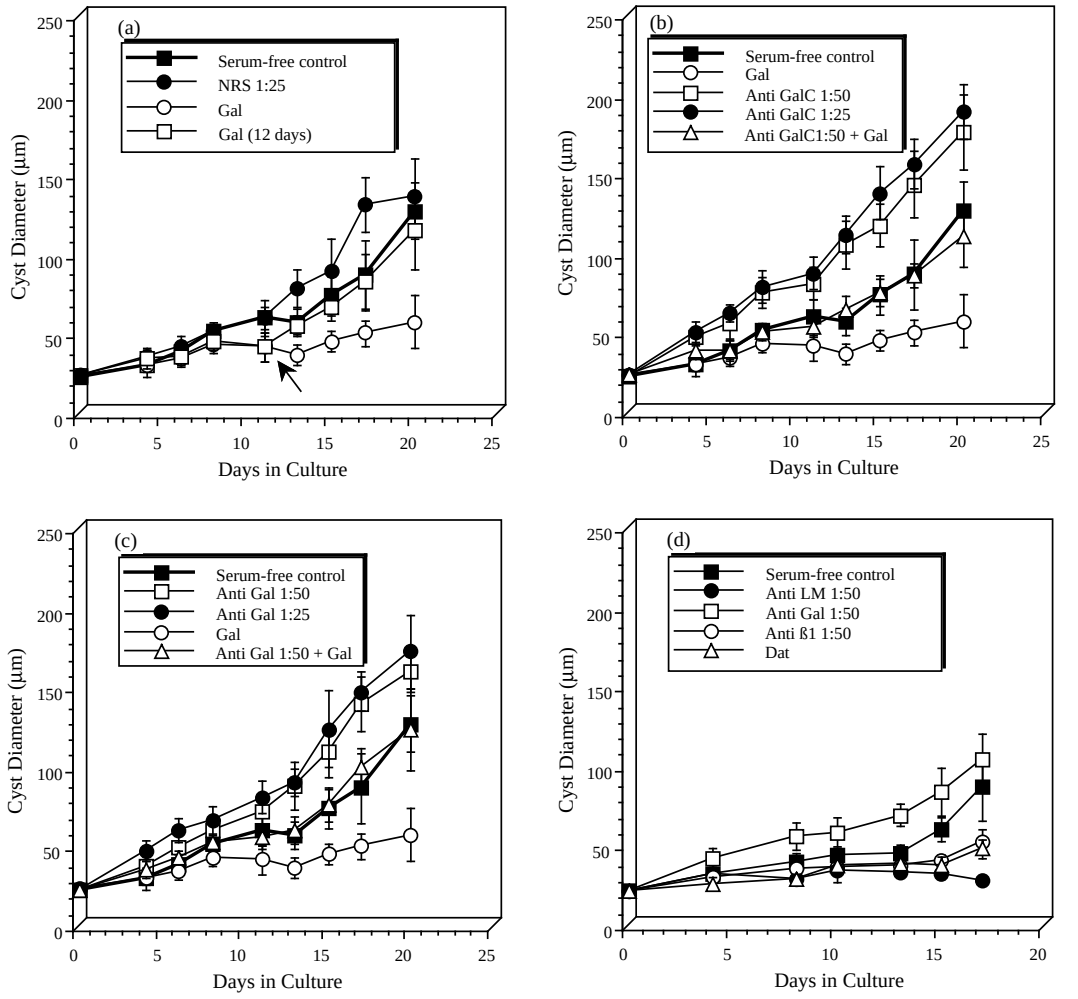

Fig. 8. Effects of lectins and antibodies on cyst enlargement by MDCK cells growing within Matrigel. Single cells seeded into Matrigel were cultured in serum-free medium or medium supplemented with various reagents and cyst size was determined as described in Materials and Methods. Each point represents the mean value of 10 measurements with vertical bars denoting standard deviation. Gal, galectin-3 added at 30 μg/ml final concentration; Anti Gal, polyclonal antibody raised against galectin-3 added at indicated dilutions; Anti GalC, polyclonal antibody raised against the C-terminal carbohydrate-binding domain of galectin-3; Anti LM, polyclonal antibody to EHS laminin; Anti β1, polyclonal antibody raised against rat liver integrin β_1 subunit; Dat, *Datura stramonium* agglutinin added at 30 μg/ml final concentration; NRS, normal rabbit serum. In one experiment (a) galectin-3 was added for 12 days in culture, then lectin-free medium (but containing Anti GalC at 1:50 dilution) was used for a further 8 days (see arrow). In others (b and c), a mixture of galectin-3 and specific antibodies was added at the start of the culture.

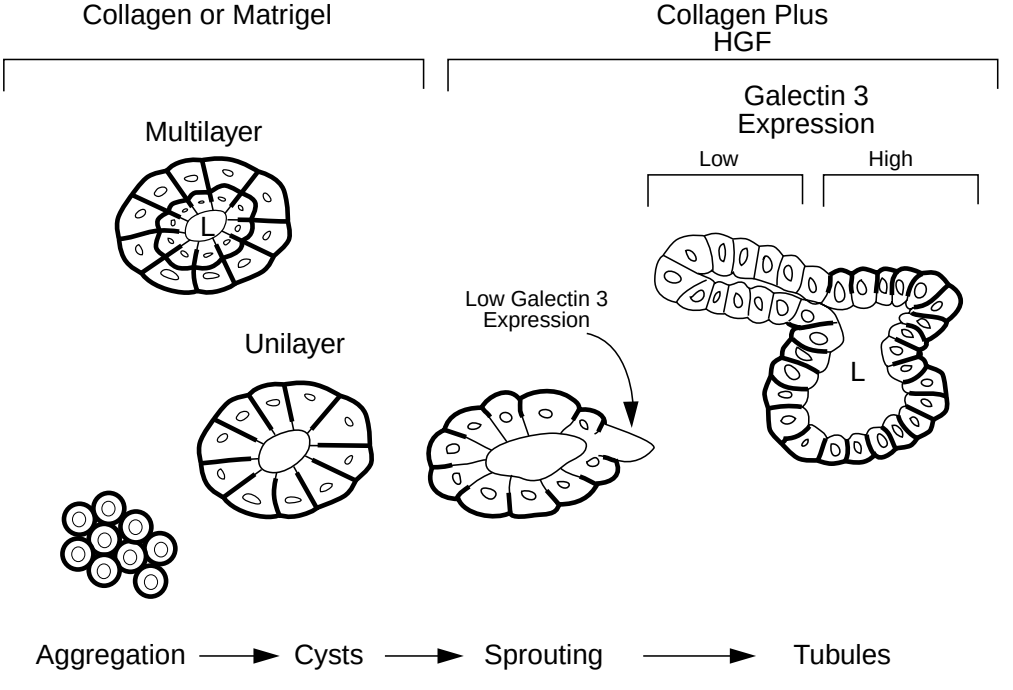

Fig. 9. Summary of galectin-3 ditribution in MDCK cells grown within collagen gels or Matrigel. Presence of the lectin at the surface of cells in loose aggregates and cysts is indicated by thick lines; lectin-free surfaces are shown by thin lines. L, lumen in cysts and tubules. Aggregation and cyst formation occur in either Matrigel or collagen gel. Sprouting and tubule formation is induced in collagen-grown cysts by HGF.

polarised cells to condensates with extensive intercellular contacts, development of polarity and formation of a polarised epithelium surrounding a central lumen and, finally, the initial events in tubule elongation stimulated by HGF. Our findings with MDCK cultures suggest that galectin-3 plays roles at the baso-lateral surfaces during these early stages and is re-targeted to apical domains once the tubular network is well established. Preliminary studies using immunofluorescence staining of hamster embryonic tissues (days 10-13) with galectin-3 antibodies does indicate a less polarised distribution of the lectin and its co-localisation with laminin to some extent (S. Bawumia and R. C. Hughes, unpublished data).

What are the roles of galectin-3 expressed at the baso-lateral surfaces in developing MDCK cysts and perhaps transiently in developing kidney epithelium? One likely role is in intercellular adhesion. Thus addition of exogenous galectin-3 to forming MDCK cysts blocks cyst expansion and galectin-3-specific antibodies accelerate expansion, results most easily explained by an enhancing or neutralising effect, respectively, on a surface function of the lectin. In solution at moderate concentration, galectin-3 is a monovalent protein (Mehul et al., 1994). However, at higher concentrations, the lectin becomes polyvalent through self-association of monomers and acts as a typical haemagglutinin (Hsu et al., 1992; Massa et al., 1993; Ochieng et al., 1993). It is possible therefore that such a bridging between glycoproteins on apposing cell surfaces could contribute to the strength of intercellular contacts. However, other mechanisms are possible. For example, galectin-3 secreted into the extracellular space may bind to other cell adhesion molecules carrying appropriate polylactosamine glycan receptors in such a way as to activate their adhesive activity. Some monoclonal antibodies to integrins are well characterised, which induce adhesive functions in resting integrin molecules (Keizer et al., 1988; van Kooyk et al., 1991; Landis et al., 1993) through binding to epitopes in extracellular domains of those molecules. Similarly, external binding to E-cadherin appears to induce association of the cytoplasmic tails of the molecule with ankyrin and stabilisation of junctional complexes (Nelson et al., 1990). Further work is required to identify the exact location of galectin-3 in lateral junctions of cyst-forming MDCK cells. Another member of the family, galectin-4, has recently been localised in adherens junctions in oral epithelium (Chiu et al., 1994).

In addition to an enhancing role in intercellular adhesions, galectin-3 at the basal surface may be involved together with laminin and β_1 integrins in cell interactions with the surrounding matrix. Such interactions appear to be important for cyst formation as shown by the inhibiting effects of laminin and integrin-specific antibodies on cyst enlargement in Matrigel. In this event, addition of large amounts of exogenous galectin-3 may overcome any adhesive function of the lectin exhibited at endogenous concentrations, similar to the inhibitory effects of high levels of galectin-3 on BHK adhesion and spreading on laminin substrata in vitro (Sato and Hughes, 1992; Hughes, 1992, 1994).

Unlike its function in cyst formation, we can find no direct role for galectin-3 in sprouting and incipient tubule formation. Addition of exogenous lectin at high concentrations or specific antibodies failed to modify the pattern or kinetics of tubule formation induced in MDCK cyst by HGF. However, it is striking that galectin-3 appears to be excluded from the tips of emerging or expanding tubules. At present, we do not know if this is due to absence of galectin-3 synthesis in cells at the leading tip of the tubule or due to an absence of suitable glycoprotein receptors on these cells for galectin-3. In either case, the exclusion of galectin-3 at these regions may be related to the need for the cells to expand into the surrounding matrix, presumably by integrin-mediated adhesions. Other factors such as type IV collagen, heparan sulphate proteoglycan and vitronectin are strong inhibitors of tubule formation by MDCK cells responding to HGF. Their mechanism of inhibition is unknown at present (Santos and Nigam, 1993).

In conclusion, the present evidence suggests roles for the polylactosamine-binding protein galectin-3 in kidney tubule morphogenesis. This protein appears to function positively in cell-cell adhesion, possibly by directly bridging surface glycoproteins on apposing cells or indirectly by strengthening other intercellular adhesion molecules. Its absence appears to be important during early tubular outgrowth, presumably because expansion requires reduced cell-cell contacts, a process that is also stimulated by HGF. Our results suggest a role for appropriate glycosylation of cell surface adhesion molecules in the developing kidney such that the surface binding of galectin-3 can be modulated, with consequent modulation of its function. Previous data also suggest the importance of protein glycosylation in kidney development as shown by the temporally and positionally specific distribution of binding sites for plant lectins with defined carbohydrate-binding specificities (Laitinen et al., 1987; Holthöfer, 1988) and the finding that interference with protein glycosylation by tunicamycin and other inhibitors prevent nephrogenesis (Ekblom et al., 1979a,b). The present results suggest one mechanism for the dependence.

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REFERENCES

Barondes, S. H., Castronovo, V., Cooper, D. N. W., Cummings, R. D., Drickamer, K., Feizi, T., Gitt, M. A., Hirabayashi, J., Hughes, C., Kasai, K., Leffler, H., Liu, F. T., Lotan, R., Mercurio, A. M., Monsigny, M., Pillai, S., Poirer, F., Raz, A., Rigby, P. W. J., Rini, J. M. and Wang, J. L. (1994). Galectins: a family of animal β galactoside-binding lectins. *Cell* **76**, 597-598.

Chiquet-Ehrismann, R. (1991). Anti-adhesive molecules of the extracellular matrix. *Curr. Opin. Cell Biol.* **3**, 800-804.

Chiu, M. L., Parry, D. A. D., Feldman, S. R., Klapper, D. G. and O'Keefe, E. J. (1994). An adherins junction protein is a member of the family of lactose-binding lectins. *J. Biol. Chem.* **269**, 31770-31776.

Cooper, D. N., Massa, S. M. and Barondes, S. H. (1991). Endogenous muscle lectin inhibits myoblast adhesion to laminin. *J. Cell Biol.* **115**, 1437-1448.

Ekblom, P., Lash, J. W., Lehtonen, E., Nordling, S. and Saxén, L. (1979a). Inhibition of morphogenetic cell interactions by 6-diazo-5-oxo-norleucine (DON). *Exp. Cell Res.* **121**, 121-126.

Ekblom, P., Nordling, S., Saxén, L., Rasilo, M-L. and Renkonen, O. (1979b). Cell interactions leading to kidney tubule determination are tunicamycin-sensitive. *Cell Differ.* **8**, 347-352.

Foddy, L., Stamatoglou, S. C. and Hughes, R. C. (1990). An endogenous carbohydrate-binding protein of baby hamster kidney (BHK21 C13) cells. Temporal changes in cellular expression in the developing kidney. *J. Cell Sci.* **97**, 139-148.

Gottari, C. and Caplan, M. J. (1993). Delivery of Na^+,K^+-ATPase in polarized epithelial cells. *Science* **260**, 552-554.

Gu, M., Wang, W., Song, W. K., Cooper, D. N. and Kaufman, S. J. (1994).

Selective modulation of the interaction of α7β1 integrin with fibronectin and laminin by L-14 lectin during skeletal muscle differentiation. *J. Cell Sci.* **107**, 175-181.

Hammerton, R. W., Krzeminski, K. A., Mays, R. W., Ryan, T. A., Wollner, D. A. and Nelson, W. J. (1991). Mechanism for regulating cell surface distribution of Na^+,K^+-ATPase in polarized epithelial cells. *Science* **254**, 847-850.

Holthöfer, H. (1988). Cell-type-specific glycoconjugates of collecting duct cells during maturation of the rat kidney. *Cell Tiss. Res.* **253**, 305-309.

Hsu, D. K., Zuberi, R. I. and Liu, F. T. (1992). Biochemical and biophysical characterization of human recombinant IgE-binding protein, an S-type animal lectin. *J. Biol. Chem.* **267**, 14167-14174.

Hughes, R. C. (1992). Lectins as cell adhesion molecules. *Curr. Opin. Struct. Biol.* **2**, 687-692.

Hughes, R. C. (1994). Mac 2: a versatile galactose-binding protein of mammalian tissues. *Glycobiology* **4**, 5-12.

Humes, H. D. and Cieslinski, D. A. (1992). Interaction between growth factors and retinoid acid in the induction of kidney tubulogenesis in tissue culture. *Exp. Cell Res.* **201**, 8-15, 1992.

Inohara, H. and Raz, A. (1994). Effects of natural complex carbohydrates on murine melanoma cell properties related to galectin 3 functions. *Glycocon. J.* **11**, 527-532.

Keizer, G. D., Visser, W., Vliem M. and Figdor, C. G. (1988). A monoclonal antibody directed against a unique epitope on the α-chain of human leukocyte function-associated antigen-1 induces homotypic cell-cell interactions. *J. Immunol.* **140**, 1393-1400.

Laitinen, L., Virtanen, I. and Saxen, L. (1987). Changes in the glycosylation pattern during embryonic development of mouse kidney as revealed with lectin conjugates. *J. Histochem. Cytochem.* **35**, 55-65.

Landis, R. C., Bennett, R. I. and Hogg, N. (1993). A novel LFA-1activation epitope maps to the I domain. *J. Cell Biol.* **120**, 1519-1529.

Lane, T. F. and Sage, E. H. (1994). The biology of SPARC, a protein that modulates cell-matrix interactions. *FASEB J.* **8**, 163-173.

Lindstedt, R., Apodaca, G., Barondes, S. H., Mostov, K. E. and Leffler, H. (1993). Apical secretion of a cytosolic protein by Madin-Darby canine kidney cells. Evidence for polarized release of an endogenous lectin by a nonclassical secretory pathway. *J. Biol. Chem.* **268**, 11750-11757.

Lotan, R., Belloni, P. N., Tressler, R. J., Lotan, D., Chun, X. X. and Nicolson, G. L. (1994). Expression of galectins on microvessel endothelial cells and their involvement in tumour cell adhesion. *Glycocon. J.* **11**, 462-468.

Mahanthappa, N. K., Cooper, D. N., Barondes, S. H. and Schwarting, G. A. (1994). Rat olfactory neurons can utilize the endogenous lectin, L-14, in a novel adhesion mechanism. *Development* **120**, 1373-1384.

Massa, S. M., Cooper, D. N., Leffler, H. and Barondes, S. H. (1993). L-29, an endogenous lectin, binds to glycoconjugate ligands with positive cooperativity. *Biochemistry* **32**, 260-267.

McAteer, T. A., Evan, A. P. and Gardner K. D. (1987). Morphogenic clonal growth of kidney epithelial cell line MDCK. *Anat. Rec.* **217**, 229-239.

Mehul, B., Bawumia, S., Martin, S. R. and Hughes, R. C. (1994). Structure of baby hamster kidney carbohydrate-binding protein CBP30, an S-type animal lectin. *J. Biol. Chem.* **269**, 18250-18258.

Mehul, B., Bawumia, S. and Hughes, R. C. (1995). Cross-linking of galectin-3, a galactose-binding protein of mammalian cells by tissue-type transglutaminase. *FEBS Lett.* **360**, 160-164.

Milos, N. and Zalik, S. E. (1983). Calcium-independent adhesion of extra-embryonic endoderm cells from the early chick blastoderm is inhibited by the blastoderm β-galactoside-binding lectin and by β-galactosidase. *Cell Differ.* **12**, 341-347.

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.

Nelson, W. J. and Hammerton, R. W. (1989). A membrane-cytoskeletal complex containing Na^+, K^+-ATPase, ankyrin, and fodrin in Madin-Darby canine kidney (MDCK) cells: implications for the biogenesis of epithelial cell polarity. *J. Cell Biol.* **108**, 893-902.

Nelson, W. J., Shore, E. M., Wang, A. Z. and Hammerton, R. W. (1990). Identification of a membrane-cytoskeletal complex containing the cell adhesion molecule uvomorulin (E-cadherin), ankyrin, and fodrin in Madin-Darby canine kidney epithelial cells. *J. Cell Biol.* **110**, 349-357.

Nigam, S. K. and Brenner, B. M. (1992). Toward an understanding of epithelial morphogenesis in health and disease. *Curr. Opin. Nephrol. Hypertension* **1**, 187-191.

Ochieng, J., Platt, D., Tait, L., Hogan, V., Raz, T., Carmi, P. and Raz, A. (1993). Structure-function relationship of a recombinant human galactoside-binding protein. *Biochemistry* **32**, 4455-4460.

Ojakian, G. K. and Schwimmer, R. (1988). The polarized distribution of an apical cell surface glycoprotein is maintained by interactions with the cytoskeleton of Mardin-Derby canine kidney cells. *J. Cell Biol.* **107**, 2377-2388.

Santos, O. F. and Nigam, S. K. (1993). HGF-induced tubulogenesis and branching of epithelial cells is modulated by extracellular matrix and TGF-β. *Dev. Biol.* **160**, 293-302.

Sato, S. and Hughes, R. C. (1992). Binding specificity of a baby hamster kidney lectin for H type I and II chains, polylactosamine glycans, and appropriately glycosylated forms of laminin and fibronectin. *J. Biol. Chem.* **267**, 6983-6990.

Sato, S., Burdett, I. and Hughes, R. C. (1993). Secretion of the baby hamster kidney 30-kDa galactose-binding lectin from polarized and nonpolarized cells: a pathway independent of the endoplasmic reticulum-Golgi complex. *Exp. Cell Res.* **207**, 8-18.

Simons, K. and Fuller, S. D. (1985). Cell surface polarity in epithelia. *Annu. Rev. Cell Biol.* **1**, 243-288.

Sorokin, L., Klein, G., Mugrauer, G., Fecker, L., Ekblom, M. and Ekblom, P. (1992). Development of kidney epithelial cells. In *Epithelial Organization and Development* (ed. T. P. Fleming), pp. 163-190. Chapman & Hall, London.

Taub, M., Wang, Y., Szczesny, T. M. and Kleinman, H. K. (1990). Epidermal growth factor or transforming growth factor α is required for kidney tubulogenesis in matrigel cultures in serum-free medium. *Proc. Nat. Acad. Sci. USA* **87**, 4002-4006.

van Adelsberg, J., Edwards, J. C., Takito, J., Kiss, B. and Al-Awqati, Q. (1994). An induced extracellular matrix protein reverses the polarity of band 3 in intercalated epithelial cells. *Cell* **76**, 1053-1061.

van Kooyk, Y., Weder, P., Hogervorst, F., Verhoeven, A. J., van Seventer, G., te Velde, A. A., Borst, J., Keizer, G. D. and Figdor, C. G. (1991). Activation of LFA-1 through a Ca^{2+}-dependent epitope stimulates lymphocyte adhesion. *J. Cell Biol.* **112**, 345-354.

Wang, A. Z., Ojakian, G. K. and Nelson, W. J. (1990a). Steps in the morphogenesis of a polarized epithelium. I. Uncoupling the roles of cell-cell and cell-substratum contact in establishing plasma membrane polarity in multicellular epithelial (MDCK) cysts. *J. Cell Sci.* **95**, 137-151.

Wang, A. Z., Ojakian, G. K. and Nelson, W. J. (1990b). Steps in the morphogenesis of a polarized epithelium. II. Disassembly and assembly of plasma membrane domains during reversal of epithelial cell polarity in multicellular epithelial (MDCK) cysts. *J. Cell Sci.* **95**, 153-165.

Woynarowska, B., Skrincosky, D. M., Haag, A., Sharma, M., Matta, K. and Bernacki, R. J. (1994). Inhibition of lectin-mediated ovarian tumor cell adhesion by sugar analogs. *J. Biol. Chem.* **269**, 22797-23803.

Yamashita, K., Totani, K., Ohkura, T., Takasaki, S., Goldstein, I. J. and Kobata, A. (1987). Carbohydrate binding properties of complex-type oligosaccharides on immobilized *Datura stramonium* Lectin. *J. Biol. Chem.* **262**, 1602-1607.

Zhou, Q. and Cummings, R. D. (1993). L-14 lectin recognition of laminin and its promotion of in vitro cell adhesion. *Arch. Biochem. Biophys.* **300**, 6-17.

Zurzolo, C. and Rodriguez-Boulan, E. (1993). Delivery of Na^+ K^+ ATPase in polarized epithelial cells. *Science* **260**, 550-552.

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