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Stage- and segment-specific expression of cell-adhesion molecules N-CAM, A-CAM, and L-CAM in the kidney

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Stage- and segment-specific expression of cell-adhesion molecules N-CAM, A-CAM, and L-CAM in the kidney. CAM expression was investigated immunohistochemically in tissue sections and in pure cultures of human proximal and distal tubular cells. In the fetal kidney, N-CAM immunoreactivity was detected in the non-induced and condensing metanephrogenic mesenchyme, and in all stages until the S-shaped bodies. A-CAM (N-cadherin) first appeared in the non-induced mesenchyme and remained present thereafter. Its expression became exclusively associated with the lower limb of the S-shaped bodies and the developing proximal tubule. In contrast, L-CAM (E-cadherin; uvomorulin) staining was observed in the fetal collecting duct, the upper limb of the S-shaped bodies, and the developing distal tubule. This segment-specific expression of A-CAM and L-CAM in the early developing nephron was maintained in the adult kidney: A-CAM staining was restricted to adherens junctions in the proximal tubule and thin limb, whereas L-CAM was expressed in Bowman's capsule and in all tubular segments except the proximal convoluted and straight tubule. Also after *in vitro* culture, A-CAM expression was an exclusive property of proximal tubular cells, while L-CAM was confined to distal tubular cells. In conclusion, each major subdivision of the fetal and adult nephron displays a characteristic combination of L-CAM and A-CAM, suggesting that they may be the basis of segmental differentiation and border formation between adjacent nephron segments.

The differentiation of dividing mesenchymal cells to an epithelial phenotype by the inductive influence of the ureteric bud and branching collecting duct is a key event during the development of the mesonephric and metanephric kidney [1]. Cell-adhesion molecules (CAMs), involved in selective cell-cell adhesion, are therefore likely to be of particular relevance to the fetal kidney. Moreover, the mammalian nephron is a complex tubular structure, composed of different epithelial cell types, grouped into sharply delineated segments which are arranged in a well-defined sequence. As all nephrons display the same basic structure, its development and preservation during adult life must be strictly regulated. Although CAMs are known to be particularly connected with pattern-formation in embryogenesis and with the maintenance of tissue pattern in adult life, their possible role in segmentation of the nephron has not been documented.

Several CAMs have been identified so far; three of them will be addressed in this study. N-CAM, originally isolated on the basis of its role in neuronal cell adhesion [2], is a Ca^{2+}-independent cell-cell adhesion molecule. L-CAM, first described by Gallin, Edelman and Cunningham [3] as a chicken liver cell-adhesion molecule and homologous to mouse uvomorulin [4], mouse E-cadherin [5], canine Arc-1 [6], and human Cell CAM 120/80 [7], provokes Ca^{2+}-dependent cell-cell adhesion. Chicken A-CAM, probably identical to chicken N-cadherin [8], is associated with intercellular adherens junctions and also mediates Ca^{2+}-dependent cell-cell adhesion. Both L-CAM and A-CAM are seen at sites of adherens junctions, but are not restricted to these areas [9].

These and other CAMs appear very early in development and are seen at various times in derivatives of all three germ layers. They regulate and are regulated by morphology [10, 11]. It has furthermore been demonstrated that CAMs are also important in pathology, such as in determining the invasive properties of epithelial cells [12]. Inversely, mouse sarcoma cells acquire epithelial characteristics by transfection with chicken L-CAM or A-CAM cDNAs [9, 13]. Moreover, expression of L-CAM in fibroblasts induces a redistribution of Na+,K+-ATPase, which becomes similar to that in polarized epithelial cells [14]. The biological importance of these molecules is further demonstrated by their well-conserved structure throughout the animal kingdom, including different classes of invertebrates [15].

The expression patterns of N-CAM, A-CAM, and L-CAM during kidney morphogenesis and in the adult organ have been described for the chick, mouse, and rat, but not in humans. In the present study, the distribution of these molecules in the human kidney was investigated immunohistochemically, and their segment-specific localization was confirmed by comparison with the distribution pattern of seven segment-specific differentiation markers covering the entire nephron. In addition, pure cultures of human proximal and distal tubular cells were analyzed for the expression of these CAMs *in vitro*. Finally, the universality of the observed expression patterns of A-CAM and L-CAM in the human kidney was assessed by comparison with a number of animal species.

Methods

Tissues

Adult normal human kidney tissue was obtained: (1) from a live 44-year-old female multi-organ donor, whose kidneys were refused for transplantation because of their spotty appearance after perfusion, and (2) at the moment of surgery from patients with renal cancer partly involving one kidney and having

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normal renal function in the absence of proteinuria ($N = 10$). The latter samples were taken at least five centimeters removed from the tumor. All samples were processed within 15 minutes after removal. Human fetal kidneys ($N = 16$) were obtained from therapeutic abortions; they were of 11, 12, 13 (2 specimens), 13.5, 14 (2 specimens), 15, 15.5, 16, 17 (2 specimens), 18, 19 (2 specimens), and 20.5 weeks gestational age. They were processed within 15 minutes after receipt. All tissues were cut into 1 to 2 mm thick slices, which were fixed during 1.5 hours at room temperature in Formol-calcium fixative [4% formaldehyde (BDH Chemical Ltd, Poole, UK) in 0.1 M sodium cacodylate buffer, pH 7.4, containing 1% $CaCl_2$], and were embedded in low-melting point (49°C) paraffin (BDH Chemical Ltd For the preparation of cryostat sections, tissue slices were snap-frozen between two copper blocks at liquid nitrogen temperature and were stored in liquid nitrogen. Formol-calcium fixed paraffin-embedded kidney tissue from a monkey (*Macaca fascicularis*), dog, mouse, and chicken were used for comparisons.

Terminology

The standard nomenclature and abbreviations for the different kidney structures, as proposed by Kriz and Bankir [16] on behalf of the Renal Commission of the International Union of Physiological Sciences, were used throughout this study. They are: CCD, cortical collecting duct; DCT, distal convoluted tubule; IM, inner medulla; IMCD, inner medullary collecting duct; ISOM, inner stripe of outer medulla; OMCD, outer medullary collecting duct; OSOM, outer stripe of outer medulla; PCT, proximal convoluted tubule, also designated as S1-S2 segments; PST proximal straight tubule, also called S3-segment; TAL, thick ascending limb; and TL, thin limb.

Culture of human proximal and distal tubular cells

Pure cultures of human PST cells and human distal tubule cells were prepared from normal outer stripe of outer medulla tissue by flow-cytometric isolation on the basis of their expression of GGT and HMFG, respectively, as described in another study of this laboratory [17]. Briefly, a single-cell suspension was prepared from minced tissue by digestion with collagenase and by isopycnic centrifugation in a discontinuous Percoll gradient. Specific fluorescent staining of the cells of interest was performed by incubation either with monoclonal antibody $102D_2K_2C_{10}$ against human kidney GGT (donated by G. Siest, University of Nancy, France) or with a mixture of the antibodies HMFG1 and HMFG2 directed against human milk fat globulin [18], followed by an indirect streptavidin-phycoerythrin staining. Cell sorting was performed using a FACS Star Plus cell sorter (Becton Dickinson Immunocytometry Systems, San Jose, California, USA). The purity after sorting was checked by flow-cytometric reanalysis and by (immuno)histochemical staining, and amounted to more than 95% for both proximal and distal tubular cells. Three day old monolayer cultures on an uncoated plastic substrate, after a total culture time of 14 days, were rinsed with PBS, fixed in Formol-calcium fixative during 10 minutes at room temperature, and stained for N-CAM, A-CAM, and L-CAM.

Immunohistochemical staining

Staining was performed on 4-micron paraffin sections, essentially as described previously [19]. Briefly, sections were mounted on poly-L-lysine (molecular wt > 300,000, Sigma Chemical Co., St. Louis, Missouri, USA) coated glass slides, hydrated, and treated for 20 minutes with 0.003% trypsin (type III, 11,250 U/mg, Sigma Chemical Co.) in 10 mM Tris-HCl buffer (pH 7.3) containing 0.9% NaCl and 1 mM $CaCl_2$. After equilibration in TBS and treatment with normal horse serum (1/5) for 20 minutes, the primary antibodies were applied without washing, and incubation was performed overnight. The sections were then washed and treated with biotinylated affinity-purified horse anti-mouse immunoglobulin serum (Vector Laboratories Inc., Burlingame, California, USA) for 30 minutes followed by the avidin/biotin/peroxidase complex (Vector Laboratories Inc.) for one hour. All dilutions were made in TBS. After extensive washing, peroxidase was revealed with 0.02% 3-amino-9-ethylcarbazole (Sigma Chemical Co.) and 0.002% H_2O_2 in 20 mM acetate buffer (pH 5.2) containing 9.5% dimethyl sulfoxide. The sections were counterstained with methyl green and mounted in Kaiser's glycerin/gelatin mounting medium.

Alternatively, 7-micron cryostat sections were air-dried and were used either as such or after immersion during five minutes in chloroform/aceton (1/1) at room temperature. They were stained according to the same procedure as paraffin sections, with omission of trypsin pretreatment.

The following mouse monoclonal antibodies were used: clone MCLA directed against human L-CAM/uvomorulin (Organon Teknika), diluted 1/50; clone DECMA-1 against mouse uvomorulin (Sigma Chemical Co.), diluted 1/4,000; clone ERIC-1 against human N-CAM (Santa Cruz Biotechnology Inc., Santa Cruz, California, USA), diluted 1/100; clone NCAM-OB11 against rat N-CAM (Sigma Chemical Co.), diluted 1/50; clone GC-4 against chicken heart A-CAM (Sigma Chemical Co.), diluted 1/100; clone IAP250 directed against human intestinal alkaline phosphatase [20], diluted 1/100; clone 14E4 directed against tissue-unspecific alkaline phosphatase from human liver, diluted 1/1,500; clones HMFG1 and HMFG2 against human milk fat globule antigen [18], culture supernatant diluted 1/100 and 1/50, respectively. Tamm Horsfall protein was visualized using a goat antiserum to human Tamm Horsfall protein (Organon Teknika, Durham, North Carolina, USA), diluted 1/100,000. In addition to the monoclonal antibodies against rat and human N-CAM, a rabbit polyclonal antiserum to rat N-CAM (Affiniti Research Products Ltd., Ilkeston, UK) was also used, diluted 1/1,000. The blocking serum and secondary antibodies were adapted accordingly.

Positive and negative control tissues for the anti-CAM antibodies comprised human jejunum, human heart, and human brain tissue, processed in an identical way to the kidney tissue.

Trypsin pretreatment of sections from paraffin-embedded tissues was essential for the demonstration of all antigens, except for N-CAM when antibody NCAM-OB11 was used. Since CAMs are sensitive to proteolytic treatment in the absence of Ca^{2+} [21], and to exclude that trypsin might have a differential effect on the release and/or degradation of L-CAM and A-CAM in different nephron segments in tissue sections from fetal and adult kidney, the trypsinization time was varied

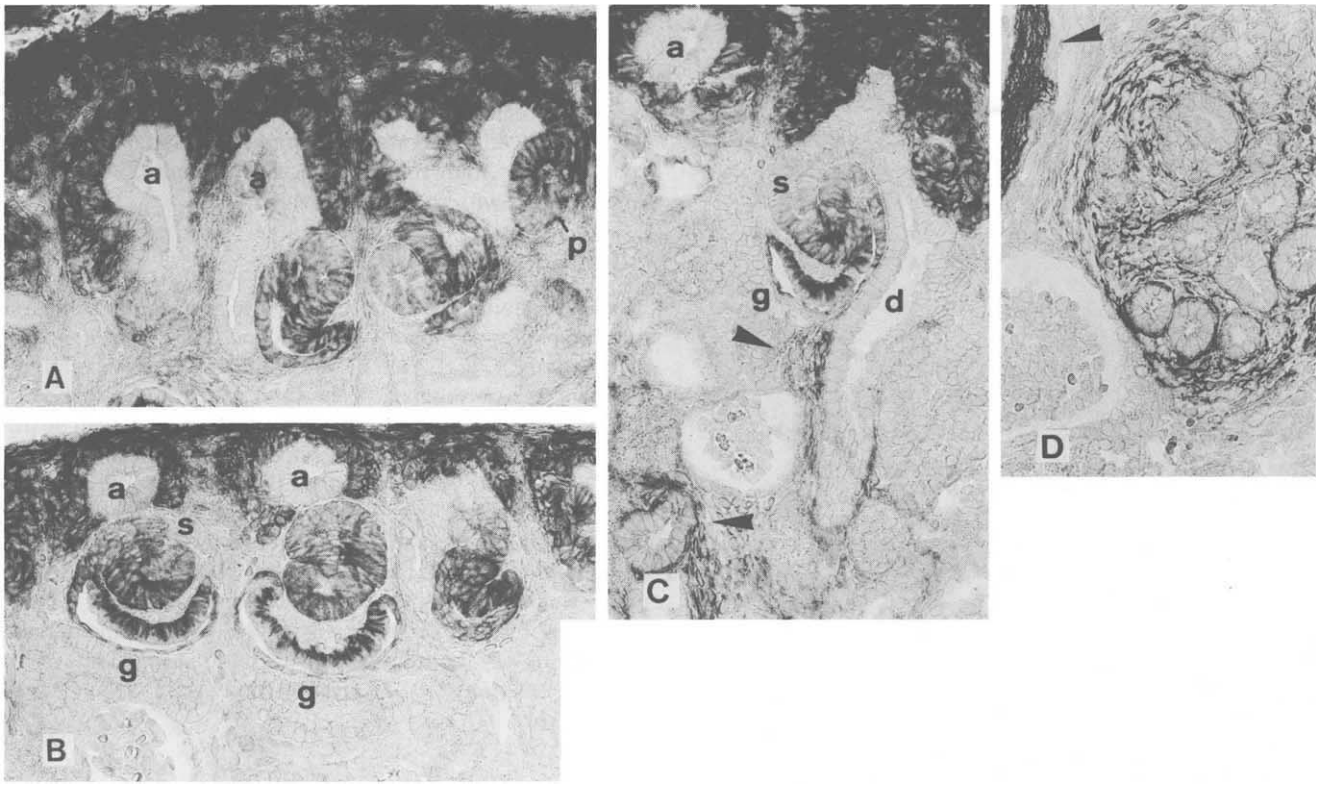


Fig. 1. *N-CAM staining (antibody ERIC-1) in 17 week fetal kidney.* **A** and **B**, strong positivity in uncondensed and condensing mesenchyme, pretubular condensate, and S-shaped bodies. **C**. S-shaped body with positive lower and middle limb connected via negative upper limb to collecting duct; arrowheads indicate areas of N-CAM positivity in stroma cells in the viscinity of some collecting ducts. **D**. Positive staining in medullary stroma; arrowhead indicates strongly positive nerve. (A-D, × 220) Abbreviations are: a, collecting duct ampulla; d, collecting duct; g, glomerulus; p, pretubular mesenchyme condensation; s, S-shaped body.

between 0 and 40 minutes, and four calcium concentrations (0, 1, 10, and 50 mM $CaCl_2$) were added to the trypsin solution.

Histochemical staining

Peanut lectin binding was performed using biotinylated peanut agglutinin (E-Y Laboratories, San Mateo, California, USA) at a concentration of 1 μg/ml, without trypsin pretreatment of the sections. Gamma-glutamyl transferase was demonstrated enzymatically, using 0.13 mg/ml gamma-glutamyl 1,4-methoxy-B-naphtylamide (Karlan Chemical Co., Santa Rosa, California, USA) as the substrate and 0.5 mg/ml Fast Blue B (Sigma Chemical Co.) as the chromogen in the presence of 0.5 mg/ml glycylglycine (Sigma Chemical Co.) in 0.03 M Tris-HCl buffer, pH 7.4. The reaction product was stabilized with 0.1 M $CuSO_4$. Alkaline phosphatase (AP) staining was performed using 5-bromo-4-chloro-3-indoxylphosphate-p-toluidine salt (Serva Feinbiochemica GmbH, Heidelberg, Germany) as the substrate and nitroblue tetrazolium (Sigma Chemical Co.) as the chromogen, according to Gossrau [22]. In addition to the immunohistochemical demonstration of IAP using monoclonal antibody IAP250, this isoenzyme was also demonstrated enzyme-histochemically by including 0.5 mM L-p-bromotetramisole in the incubation mixture [23], an inhibitor of the tissue-unspecific isoenzymes of AP [24]. Some fetal tissue sections were double-stained for both AP (dark blue reaction product) and for L-CAM or A-CAM (red reaction product).

Results

Specificity of the antibodies

N-CAM staining was strong in human brain tissue, but was absent in jejunum (except strong staining in nerve fibers) and heart. Strong staining was also seen in small and larger nerves in the developing (Fig. 1D) and adult kidney. In the positive control tissue for A-CAM, that is, adult human heart, strong staining was localized on the intercalated discs of the muscle cells (Fig. 2); there was no staining in jejunum or brain. Finally, L-CAM staining in jejunum was strong on the apical sites of contact between the epithelial cells while moderately strong staining was seen on the lateral and occasionally also on the basal plasma membrane (Fig. 3). Staining for L-CAM was absent in heart and brain.

Variations in the duration of trypsin treatment of sections had no effect on the topographic distribution pattern for A-CAM and L-CAM in the fetal and adult kidney. The intensity of staining was highest for both antigens after 20 minutes of treatment. According to several reports [25, 26], inclusion of 1 mM $CaCl_2$ in the trypsin solution, as is routinely used in our laboratory [19], is sufficient to reach a plateau in the protective effect of Ca^{2+} on the tryptic degradation of Ca^{2+}-dependent CAMs. This was confirmed by comparing the staining levels for L-CAM and A-CAM in human fetal and adult kidney sections

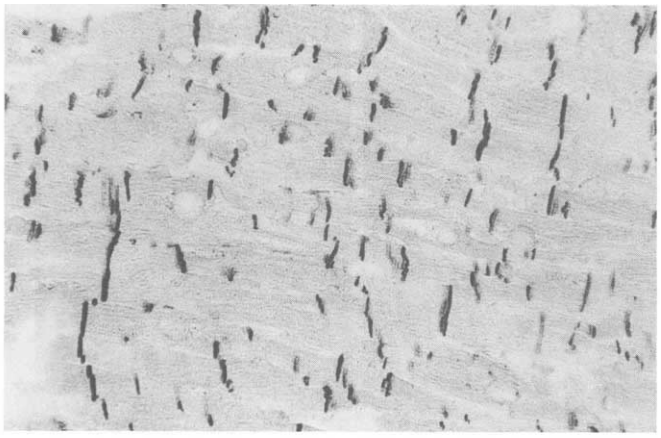


Fig. 2. *A-CAM staining in human heart muscle (× 275).*

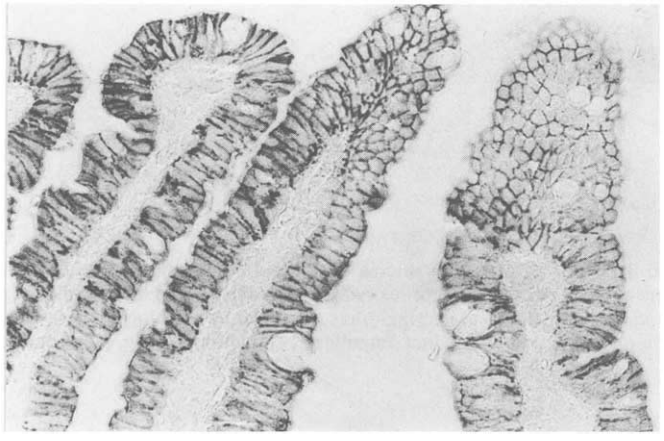


Fig. 3. *L-CAM staining (antibody MCLA) in human jejunum (× 275).*

that had been pretreated with thrypsin containing 0, 1, 10, or 50 mM Ca^{2+}.

Fetal kidney

The expression patterns for N-CAM, A-CAM, and L-CAM in different stages of nephron development are summarized in Figure 4.

N-CAM

Clone ERIC-1 against human N-CAM, clone NCAM-OB11 against rat N-CAM, and the polyclonal antiserum against rat N-CAM gave qualitatively the same staining patterns. Nevertheless, antibody ERIC-1 performed substantially better than the other two reagents. Strong staining was observed on the non-induced mesenchyme in the subcapsular area (Fig. 1 A-C) and in the sheets of Bertin, in the early condensed mesenchyme around the collecting duct endings (Fig. 1 A and B), the isolated spherical cell masses (futher referred to as pretubular condensates) (Fig. 1 A and C), the comma-shaped bodies, and the lower and middle limb of the S-shaped bodies (Fig. 1C). During early glomerular development, the visceral and parietal glomerular epithelium were both positive (Fig. 1 B and C); in the former epithelium, positivity was mainly seen at the basis of the cylindrical cells, whereas in all previous stages the entire cell surface was positive. Further developmental stages were completely negative. In addition, N-CAM staining was also observed with each antibody in the tubulointerstitial cells of the medullary region (Fig. 1 C and D). Although antibody NCAM-OB11 produced moderate staining on the luminal surface of the epithelial cells lining the collecting duct ampullae, this could not be confirmed with the other antibodies.

A-CAM

A-CAM staining was observed in all stages of metanephric development, starting in the uncondensed and condensing mesenchyme (Fig. 5A). The following stages of development, including the pretubular condensates, the comma-shaped bodies, and the S-shaped bodies (Fig. 5 A and C), were all positive. In the latter, however, staining was restricted to the lower limb, that is, the future proximal tubule (Fig. 5 B and C). In the early developing glomerulus, A-CAM was seen in the parietal epithelium and also at the antiluminal side of the cylindrical visceral epithelium (Fig. 5 A and B). In all further stages, A-CAM expression was restricted to the developing proximal tubule, as evidenced by its overlapping with the histochemical staining patterns for AP, the staining restricted to the apical intercellular contact-sites (Fig. 5 D). Collecting ducts were always negative for A-CAM.

L-CAM

The entire collecting duct system, including the growing bud, was strongly L-CAM positive (Fig. 6 A and B). Staining was absent in the undifferentiated metanephrogenic mesenchyme, also after its condensation around the collecting duct endings (Fig. 6 C-E) and its conversion into pretubular condensates and comma-shaped bodies (Fig. 6C). L-CAM positivity first appeared in the upper limb of the S-shaped bodies (Fig. 6D and E) and continued to be present in the distal part of the developing nephron (Fig. 6B). Staining was localized on the entire cell surface of all cells lining the collecting ducts and developing distal tubules. Combination of L-CAM immunohistochemical staining with histochemical staining for total AP revealed that they were mutually exclusive, except in the upper limb of the S-shaped bodies, which demonstrates the absence of L-CAM in the developing proximal tubule. The developing glomerular tuft was negative (Fig. 6 C-E), but in later stages the lateral plasma membranes of the parietal epithelial cells lining Bowman's capsule were occasionally positive.

IAP

In contrast to the adult kidney, intestinal alkaline phosphatase (IAP) staining could not be detected in the developing kidney between 11 and 21 weeks gestation, neither immunohistochemically using monoclonal antibody Mab 250, nor enzyme-histochemically using 0.5 mM L-p-bromotetramisole to inhibit the tissue-unspecific isoenzyme of AP. The absence of IAP in all fetal kidneys investigated probably is due to a lack of functional and morphological specialization between the early and the late part of the proximal tubule at these stages of development, hereby confirming our previous conclusion that IAP in the human kidney is a differentiation marker for the S3-segment [20].

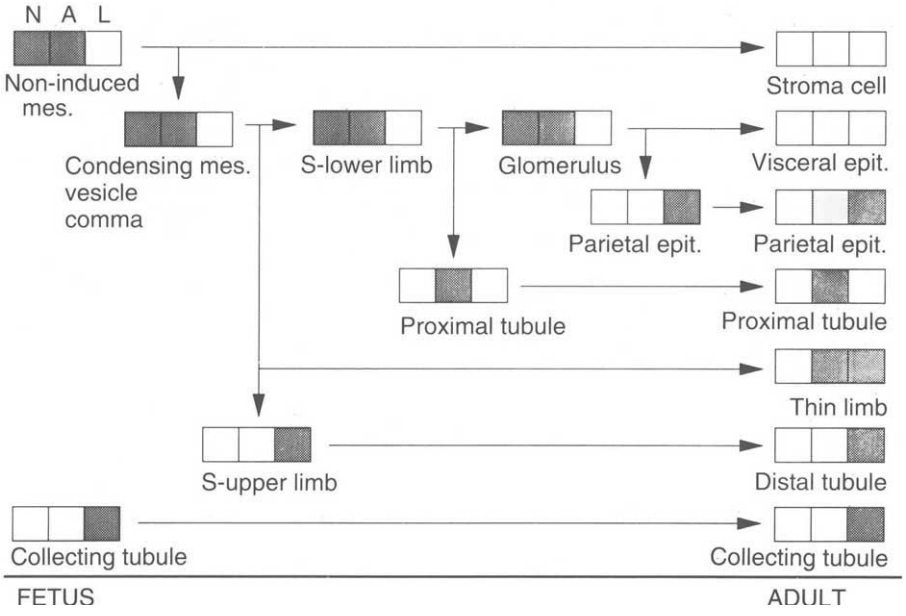


Fig. 4. *Switches in N-CAM (left squares), A-CAM (middle squares), and L-CAM (right squares) expression during consecutive stages of human metanephric development.* Darkening of squares indicates the expression level.

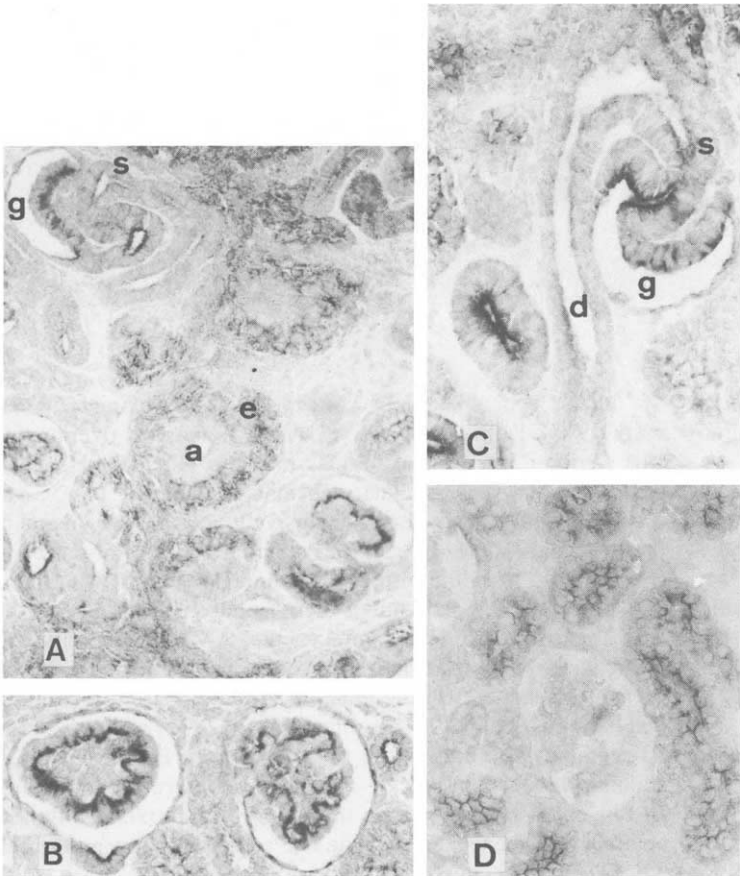


Fig. 5. *A-CAM staining in 21 week fetal kidney.* **A.** Sheet of Bertin showing positivity in uncondensed and condensing mesenchyme, visceral glomerular epithelium, and lower limb of S-shaped body. **B.** Positive staining at the antiluminal surface of visceral glomerular epithelial cells, in the parietal epithelium, and at the luminal surface of proximal tubular cells lining the urinary pole. **C.** S-shaped body connected to collecting duct, displaying positivity in visceral epithelium and lower limb. **D.** Staining at the luminal sites of intercellular contact in maturing proximal tubule. (A-D, × 220) Abbreviations are: a, collecting duct ampulla; d, collecting duct; e, early condensing mesenchyme; g, glomerulus; s, S-shaped body.

Adult kidney

No differences in the staining patterns for any of the CAMs were seen between the donor kidney and the normal tissues that were taken from tumor-affected kidneys. The staining patterns for N-CAM, A-CAM, and L-CAM, and for seven markers that are more or less specifically expressed in different nephron segments are summarized in Table 1. The following markers were used: the tissue-unspecific isoenzyme of alkaline phosphatase (TUAP) and gamma-glutamyl transferase (GGT) as markers for the proximal tubule [27], the intestinal-type isoenzyme of alkaline phosphatase (IAP), exclusively expressed in

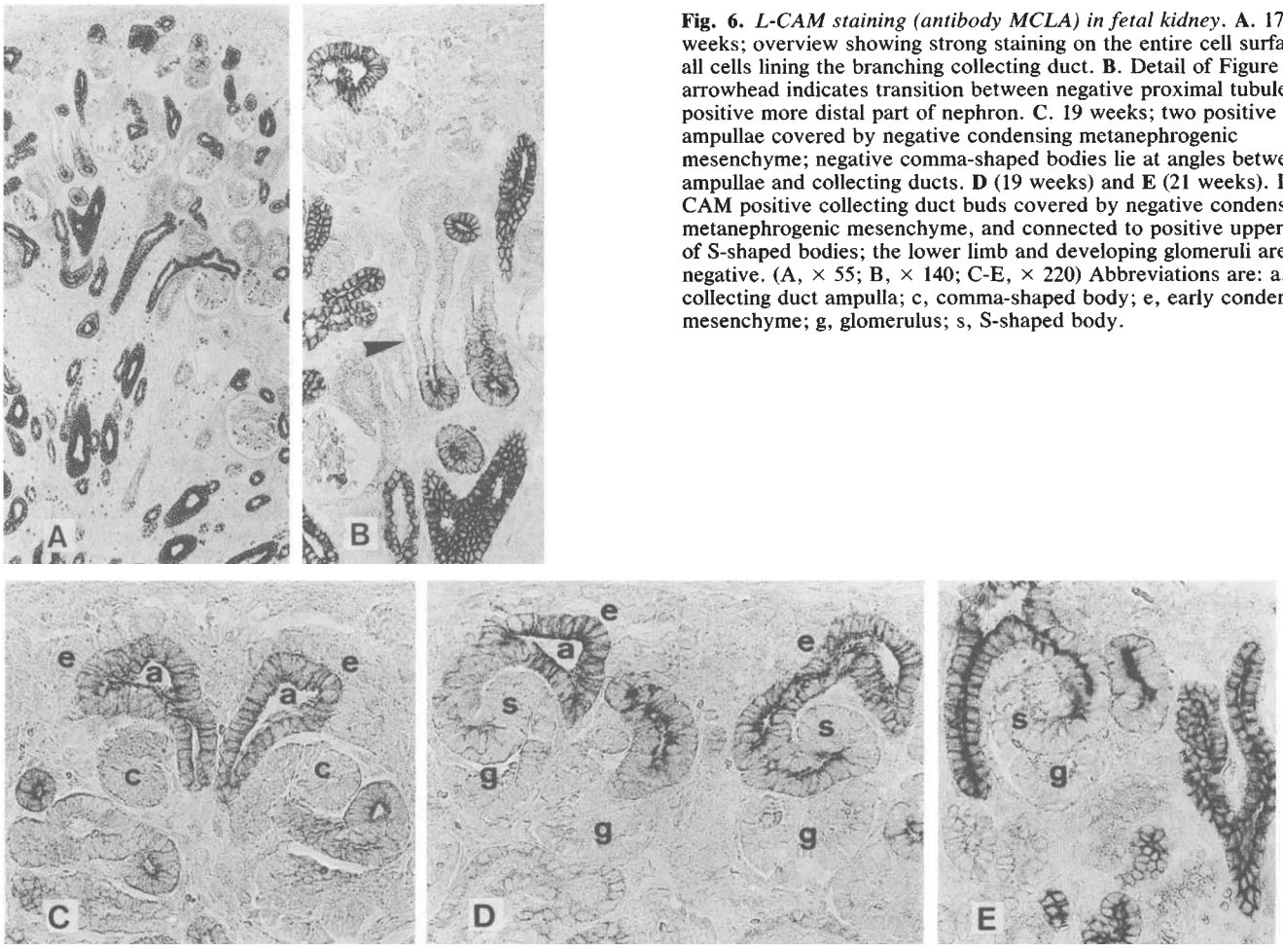


Fig. 6. *L-CAM staining (antibody MCLA) in fetal kidney.* **A.** 17 weeks; overview showing strong staining on the entire cell surface of all cells lining the branching collecting duct. **B.** Detail of Figure A; arrowhead indicates transition between negative proximal tubule and positive more distal part of nephron. **C.** 19 weeks; two positive ampullae covered by negative condensing metanephrogenic mesenchyme; negative comma-shaped bodies lie at angles between ampullae and collecting ducts. **D** (19 weeks) and **E** (21 weeks). L-CAM positive collecting duct buds covered by negative condensing metanephrogenic mesenchyme, and connected to positive upper limb of S-shaped bodies; the lower limb and developing glomeruli are negative. (A, × 55; B, × 140; C-E, × 220) Abbreviations are: a, collecting duct ampulla; c, comma-shaped body; e, early condensing mesenchyme; g, glomerulus; s, S-shaped body.

the straight segment of the proximal tubule (PST) [20], Tamm-Horsfall protein (THP) as a marker for the thick ascending limb (TAL) [28], human milk fat globule antigen (HMFG) [18], homologous to epithelial membrane antigen (EMA), and peanut lectin (PNA) binding capacity, both present along the entire distal nephron. The CAM expression patterns are schematically represented in Figure 7.

The staining patterns for A-CAM and L-CAM on paraffin sections were identical to those obtained on unfixed or chloroform/aceton-fixed frozen sections.

N-CAM

When antibody ERIC-1 against human N-CAM or the rabbit antiserum against rat N-CAM were used, no staining could be detected in the adult kidney, except for interstitial nerve fibers and nerves, which were strongly positive. With antibody NCAM-OB11 against rat N-CAM, some TL cross-sections in the ISOM and IM contained a number of cells displaying strong N-CAM staining on their apical cell surface, and some glomeruli contained a few weakly stained cells.

A-CAM

A-CAM positive staining in the cortex and medullary rays (Fig. 8 A and B), and in the OSOM co-localized completely with the histochemical staining pattern for the proximal tubular markers GGT (Fig. 8C) and TUAP, whereas no overlapping was seen with the distal tubular markers HMFG2, HMFG1, THP, and PNA. This illustrates that in these areas A-CAM immunoreactivity was found exclusively and in all segments of the proximal tubule. In addition, staining was also observed in the thin loop of Henle in the ISOM and IM (Fig. 8D). Staining was present in all cells lining these nephron segments. In the S1-S2 segments of the proximal tubule, A-CAM positivity was particularly evident at the apical sites of intercellular contact, presumably adherens junctions, and in an apparently continuous band just below the brushborder (Fig. 8A), whereas in the S3-segment (Fig. 8A) and in the thin loop of Henle (Fig. 8D), staining was seen only at the apical sites of intercellular contact. In tangential cross-sections through S1-S2 proximal tubules, staining was seen as zig zagging line segments between the cell apices (Fig. 8A). In contrast, in the S3-segments it consisted of straight line segments (Fig. 8A). In addition to the strong apical staining, there was substantially weaker staining associated with the basal cell surface of proximal tubular cells. Weak staining was also seen at the intercellular contact sites in Bowman's capsule. The described A-CAM staining pattern was present in all kidneys investigated, but, like for L-CAM, there was considerable variation in the intensity. The staining levels

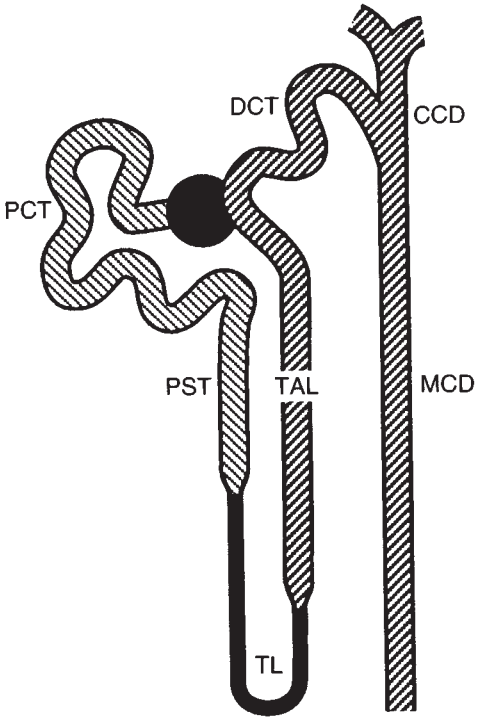


Fig. 7. *Schematic representation of the segment-specific expression of A-CAM and L-CAM along the adult human nephron.* Symbols are: (▧) A-CAM, (▨) L-CAM, (■) both.

for both antigens were positively correlated and they also seemed to be correlated with the quality of tissue preservation.

In the other mammalian species investigated, A-CAM could not be demonstrated with antibody GC-4 against chicken A-CAM. In the chicken, strong staining was seen only in the connective tissue capsula and septa (data not shown).

L-CAM

All specimens of human kidney were analyzed using antibody MCLA against human L-CAM; some were also stained with antibody DECMA-1 against mouse uvomorulin. The obtained staining patterns were identical, apart from a slightly higher level of staining with the former antibody. All tubular cross-sections in which L-CAM staining could be detected were also positive for HMFG2 and for PNA, in the cortex (Fig. 9 A-C), medullary rays and OSOM, ISOM, and IM. The thin limb was L-CAM positive (Fig. 9D), but HMFG2 negative. All HMFG1-immunoreactive DCT and collecting ducts were L-CAM positive as well. Furthermore, also all THP positive TAL cross-

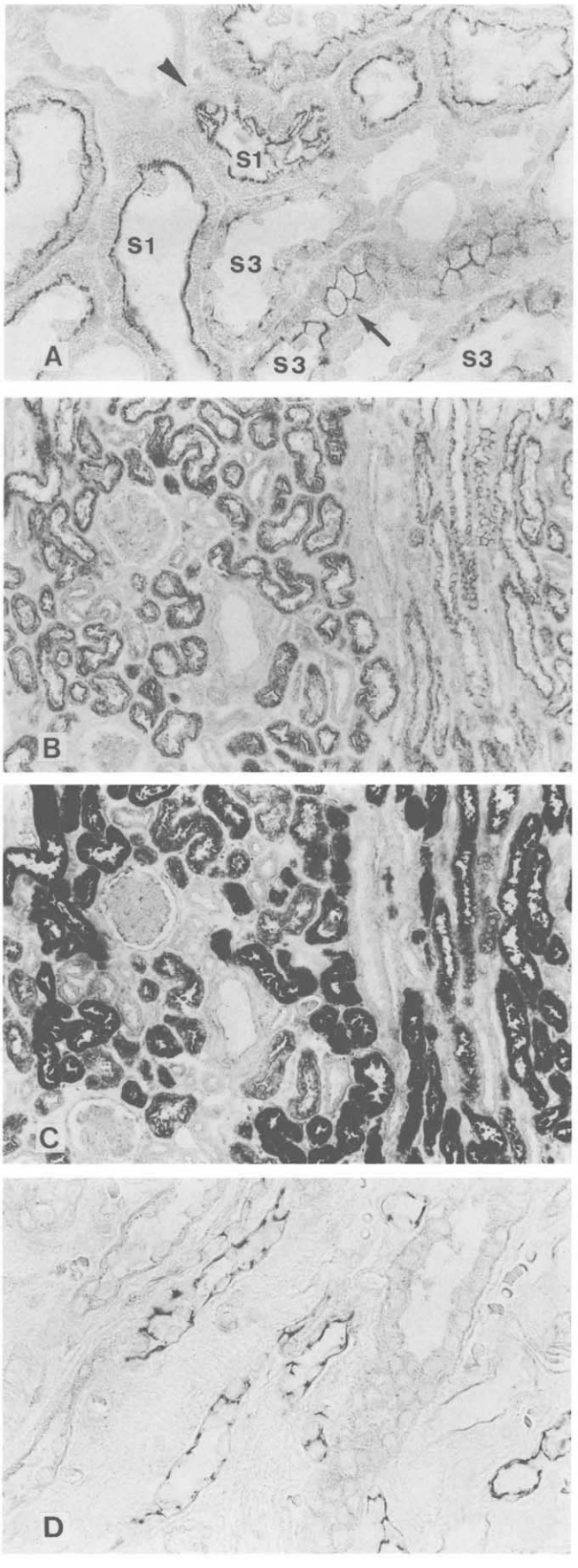
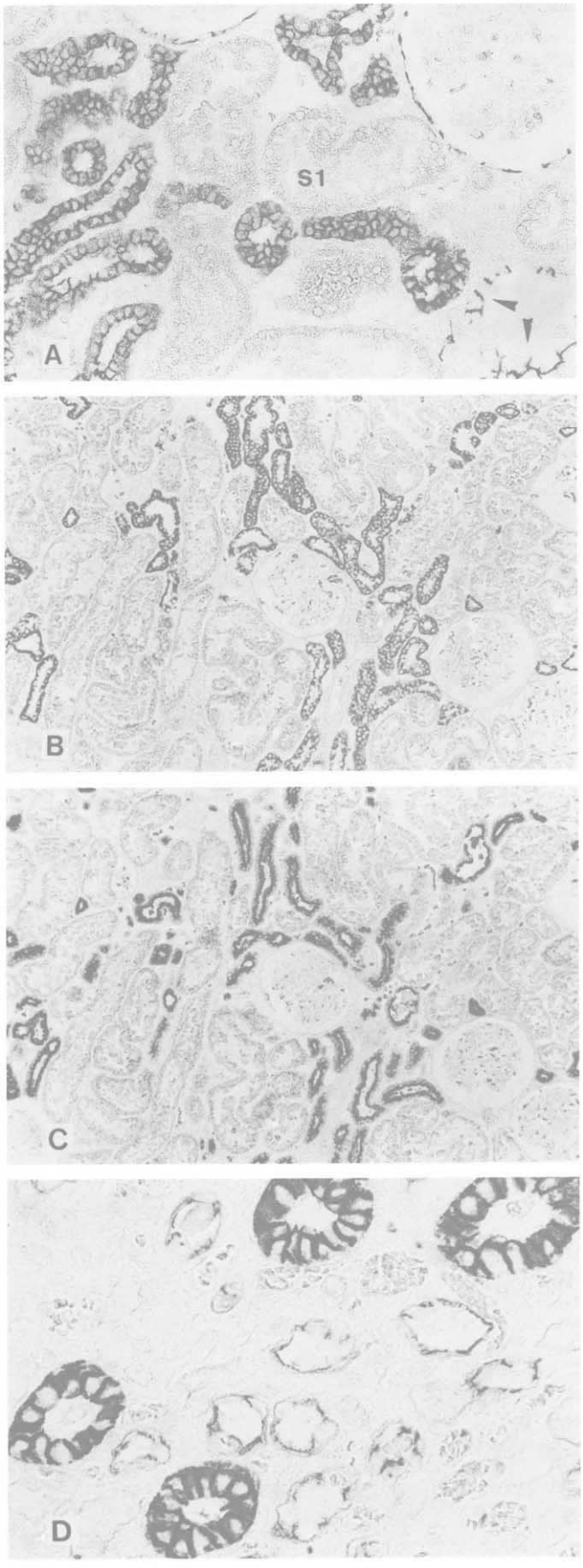


Fig. 8. *A-CAM staining in adult human kidney.* **A.** Difference in A-CAM localization between S1-S2 and S3 proximal tubular cells. Tangential tubular cross-sections show staining as zig-zagging line segments along cellular interdigitations between S1-S2 cells (arrowhead), in contrast to the straight line segments between S3-cells which lack intercellular interdigitations (arrow). **B** and **C.** Adjacent sections containing cortex and medullary ray. B, A-CAM staining; C, GGT histochemical staining that completely overlaps with the staining in B; D, A-CAM staining in TL segments of inner stripe of outer medulla. (A, × 275; B-C, × 70; D, × 175)



sections in the OSOM and ISOM were L-CAM positive. These comparisons demonstrate that L-CAM expression was detected in all segments downstream to the proximal tubule, that is, from the thin limb to the inner medullary collecting duct, including the entire distal nephron (Table 1). This L-CAM staining pattern was consistently observed in the eleven specimens investigated, although there was considerable variation in the staining intensity. When staining was strong, it was present on the entire cell surface of the tubuloepithelial cells; when it was less intense, it was predominantly localized at the apical sites of intercellular contact. These patterns resemble the localization of staining in jejunal epithelial cells (vide supra; Fig. 3). No overlapping existed in cortex and OSOM between L-CAM staining and staining for GGT, TUAP, or IAP, illustrating that L-CAM is not expressed in the S1, S2 and S3-segments of the proximal tubule. IAP was present exclusively on the brush border of the epithelial cells lining the S3-segment of the proximal tubules in the medullary rays and OSOM, as described previously [17]. Finally, positive L-CAM staining was also present on the lateral membranes of the parietal epithelial cells of the Bowman's capsule (Fig. 9A).

This absence of L-CAM expression in the proximal convoluted and straight tubule was also seen in the monkey (antibodies MCLA and DECMA-1) and dog kidney (antibody DECMA-1; data not shown). In contrast, in the mouse, L-CAM staining (antibody DECMA-1) was seen both in proximal and distal tubular cross sections; staining was particularly strong in collecting ducts, mainly along the basolateral cell surfaces (data not shown). No staining could be obtained in the chicken kidney.

Cell cultures from adult kidney

Cell cultures from distal tubular origin were positive for L-CAM (Fig. 10), and negative for A-CAM, like their *in vivo* counterparts. In analogy, cultures of proximal tubular cells were positive for A-CAM and negative for L-CAM (Fig. 11). Positive staining in both types of cultures was found as line segments along the lateral plasma membranes of neighbouring cells. N-CAM could not be detected in either type of culture.

Discussion

Because of their specific adhesive properties, CAMs are potential effectors of morphogenesis. However, since the addition of an excess of neutralizing antibodies to N-CAM or L-CAM failed to inhibit the major events in embryonic kidney development [29, 30], their biological significance in nephrogenesis has been questioned. Nevertheless, similar experiments revealed their importance in cell-adhesion in preimplantation mouse embryos [4, 7, 31] and in the morphogenesis of other organ systems, such as during feather development of chick embryo skin *in vitro* (32), motor neuron migration *in vitro* [33], and the alignment of cell layers in neural retina [11].

Fig. 9. *L-CAM staining (antibody MCLA) in adult human kidney.* **A.** Positive staining at the lateral cell contacts in parietal epithelium of Bowman's capsule (small arrowheads) and in the DCT and CCD; absence of staining in the PCT (S1). **B-C.** Comparison on adjacent sections in cortex of L-CAM staining (B) with the distribution pattern for the distal tubular marker HMFG2 (C). **D.** L-CAM staining in thin limbs and collecting ducts in inner medulla. (A, × 175; B-C, × 70; D, × 275)

Table 1. Comparison of the staining pattern for N-CAM, A-CAM, and L-CAM in the adult human kidney with the distribution of seven segment-specific markers

Zone	Segment	N-CAM	A-CAM	L-CAM	HMFG2	PNA	HMFG1	THP	GGT	TUAP	IAP
Cortex	GLOM	−	+	++	−	−	−	−	−	−	−
	PCT	−	+++	−	−	−	−	−	+	+++	−
	TAL	−	−	++	+++	+++	±	+++	−	−	−
	DCT	−	−	++	+++	++	+++	−	−	−	−
	CCD	−	−	++	+++	+++	+++	−	−	−	
OSOM	PST	−	++	−	−	−	−	−	+++	++	++
	TAL	−	−	++	+++	+	−	+++	−	−	−
	OMCD	−	−	+++	+++	++	+++	−	−	−	−
ISOM	TL	−	++	++	−	±	±	−	−	−	−
	TAL	−	−	++	+++	+	−	+++	−	−	−
	OMCD	−	−	+++	+++	++	+++	−	−	−	−
IM	TL	−	++	++	−	±	±	−	−	−	−
	IMCD	−	−	++	++	++	+++	−	−	−	−

Abbreviations are: CCD, cortical collecting duct; DCT, distal convoluted tubule; GGT, gamma-glutamyl transferase; GLOM, glomerulus; HMFG, human milk fat globulin; IAP, intestinal-type alkaline phosphatase; IM, inner medulla: IMCD, inner medullary collecting duct; ISOM, inner stripe of outer medulla; OMCD, outer medullary collecting duct; OSOM, outer stripe of outer medulla; PCT, proximal convoluted tubule; PNA, peanut agglutinin binding; PST proximal straight tubule; TAL, thick ascending limb; THP, Tamm-Horsfall protein; TL, thin limb; and TUAP, tissue-unspecific alkaline phosphatase.

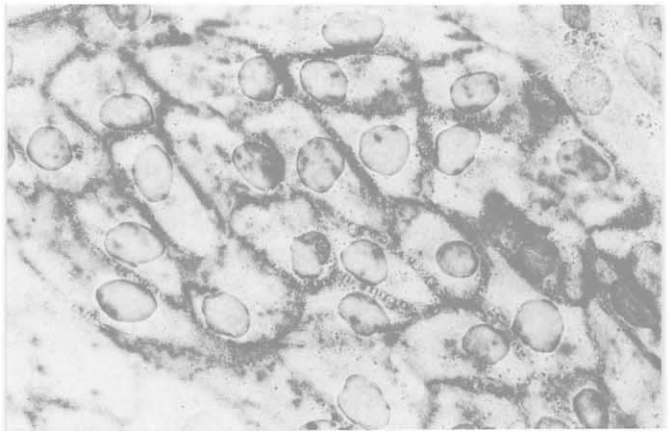


Fig. 10. *L-CAM staining (antibody MCLA) in confluent cultures of human distal tubular cells (× 550).*

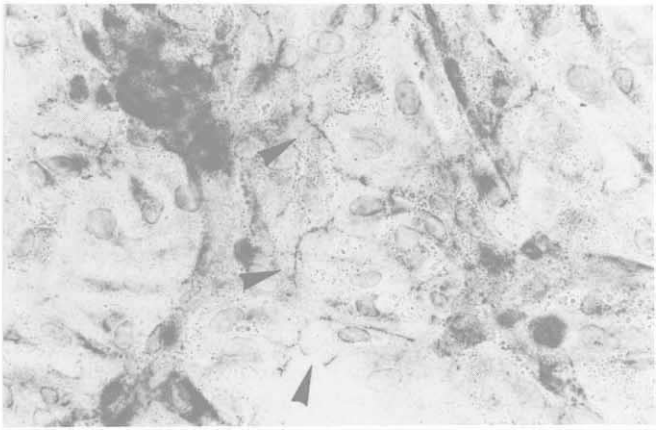


Fig. 11. *A-CAM staining in confluent cultures of human proximal tubular cells (× 275).*

Since N-CAM is present in both non-induced and condensing metanephrogenic blastema cells, it is not very likely to be the adhesive factor responsible for this condensation. Moreover, N-CAM remains present in fetal medullary interstitial cells, for which there is no reason to suppose that they could be involved in cell condensation at a later stage. Also L-CAM poses some problem in this regard, as its expression is detected rather late after completion of the epithelial conversion, that is, early before the upper limb of the S-shaped body, that will further develop into the distal tubule, merges with the collecting duct. The biological rationale for the switch to L-CAM expression in the upper limb may be to promote this fusion through the expression of the same type of CAM as in the other fusion partner, the L-CAM positive collecting duct. As A-CAM, like N-CAM, is expressed both in the non-induced and condensing mesenchyme, its mere presence is again unlikely to be responsible for the mesenchyme-to-epithelium conversion. However, its expression from the very beginning and throughout human nephron development is interesting, and its exact role during kidney morphogenesis should be further investigated. A-CAM was first isolated from purified chick cardiac intercalated discs [34] and is structurally related to L-CAM. Conflicting data exist on whether or not they interact in a heterotypic manner [9, 35].

Our results on the expression of N-CAM, A-CAM, and L-CAM in the fetal human kidney are in general in agreement with available data for a few other species, but there are nevertheless some important differences. Our N-CAM expression pattern is identical to that described by others for the developing meso- and metanephric avian kidney [36] and for the metanephric mouse kidney [37]. Comparison between our A-CAM data and the described pattern for A-CAM [8] and N-cadherin [38] in the developing meso- and metanephric chicken kidney reveals two important dissimilarities. In addition to the A-CAM positivity as seen in the human kidney, the chicken displays staining in the Wolffian duct and the collecting ducts. Moreover, A-CAM is gradually replaced by L-CAM in the maturing nephron, first in the proximal and subsequently in the distal tubules. Finally, our L-CAM staining pattern is essentially the same as in the chicken embryonic kidney, except that in the latter all developing metanephric tubules become

positive [39]. A similar pattern was described during earlier mesonephric development [36, 39, 40]. Also, in the fetal mouse a distinct reaction was reported to be present on all cells both in the proximal and the distal tubules, in contrast to the human fetal kidney, and no reaction could be detected at any stage within the developing glomeruli [41].

The complementary segment-specificity in the expression of A-CAM and L-CAM that becomes apparent very early in the developing human nephron was also recognized in the adult human kidney, where it was further evidenced by comparisons with staining patterns obtained for seven segment-specific markers covering the entire nephron. L-CAM staining on the one side was restricted to cells lining the entire distal nephron, from the thin limb in the inner stripe of the outer medulla to the collecting duct in the inner medulla, and in Bowman's capsule. An exclusive distal tubular expression of L-CAM could also be detected in the monkey and dog kidney, but not in the mouse, which illustrates that the human expression pattern is not an exception. Moreover, this association between L-CAM expression and the distal nephron was conserved after in vitro culture of human distal tubular cells for two weeks. Similarly, the MDCK dog kidney cell line, displaying a number of distal tubular characteristics, is also known to express uvomorulin [42]. These data demonstrate that L-CAM is an important differentiation marker for all nephron segments downstream to the proximal tubule. A-CAM staining, on the other hand, was detected only in the proximal tubule and thin limb, and to a lesser extent in the epithelium lining Bowman's capsule. Staining was predominantly associated with the apical zones of intercellular contact, presumably adherens junctions. Again, the exclusive association between the expression of A-CAM and the proximal nephron was conserved after in vitro culture of human proximal tubular cells for two weeks.

As for the fetal kidney, there are similarities and differences between our CAM staining patterns in the adult human kidney and those reported for three animal species.

The absence of N-CAM is in agreement with available data for the mouse, where N-CAM and its mRNA are lost during kidney development [37].

A-CAM staining has been previously reported only for the human and chicken kidney. Using clone GC-4 on frozen sections, A-CAM has been detected on the basolateral surface of the tubular epithelial cells throughout the length of the nephron in the adult human kidney [43]. This is in contradiction with our data for the same antibody on formalin-fixed paraffin-embedded tissue and on frozen sections as well. However, the reliability of our A-CAM stainings is supported by the following features: (1) staining in both the fetal and adult proximal tubule is found only on the apical contact sites between the cells, as is to be expected for an adherens-junction-specific cell adhesion molecule; (2) the difference in A-CAM staining between S1-S2 and S3 proximal tubular cells in the adult kidney is in accordance with known differences in the amount of adherens-junction components between these cells, as lateral interdigitations are abundant between S1-S2 cells and are absent between S3-cells [44]; (3) A-CAM expression *in vitro* is again restricted to cells of proximal tubular origin; (4) staining in human heart is restricted to the intercalated discs of muscle cells. Our inability to detect A-CAM in tubules of the adult chicken kidney is in accordance with the lack of staining reported by others [8].

As for L-CAM, Biddlestone and Fleming [43] were unable to demonstrate this molecule on frozen sections of adult human kidney, using monoclonal antibody DECMA-1 against mouse uvomorulin. In contrast, we could demonstrate L-CAM in the distal nephron using this antibody and also using the anti-human L-CAM antibody MCLA on frozen as well as on paraffin sections. The absence in our study of L-CAM in the proximal tubule also is in disaggreement with the available information on the chicken [39, 40], mouse and rat [31, 41]. In the chicken, L-CAM staining has been observed in all tubule structures and on the parietal cells of Bowman's capsule [39]. In the adult mouse [31] and rat [41], positive L-CAM staining was localized mostly on epithelia of the collecting tubules. In another study, the authors report that both proximal and distal tubules in adult mouse are positive for L-CAM, but that they are more weakly stained than the collecting ducts [29]. Glomeruli were described to be negative. We could confirm this expression pattern by using antibody DECMA-1 on formalin-fixed paraffin-embedded mouse kidney tissue. This proves that the disparities in the L-CAM staining pattern between human and mouse reflect a biological reality and are not the consequence of differences in methodologies used. Finally, E-cadherin has also been demonstrated in kidney tubules from *Xenopus laevis* [45]; staining was found in the Wolffian duct and some mesonephric tubules, although most tubules of the early mesonephros were negative.

It is intriguing that tubuloepithelial differentiation along the human nephron is accompanied by the segment-specific expression of the cell adhesion molecules A-CAM and L-CAM. This might indicate that the functional load of discrete nephron segments imposes different demands on mechanical linkage between adjacent cells. Alternatively, it could be a means to establish during development, and to conserve thereafter, the sharp borders between nephron segments, that is, to prevent the intermingling of different cell types at the transition between segments. The latter explanation seems more plausible, since the segment-specific CAM expression is already installed from the very beginning of segment-specific development of the nephron (the S-shaped body), that is, before the acquisition of functionality, and also since it is conserved *in vitro* in conditions in which a normal functioning of the cells is unlikely to occur. Moreover, there is an abrupt switch in the expression of L-CAM and/or A-CAM at each sharp morphological border between adjacent parts of the nephron, that is, between Bowman's capsule and the proximal tubule, between the proximal tubule and the thin limb, and between the thin limb and the distal tubule. No information is available on the mechanisms by which these sharp morphological transitions between adjacent segments could be installed and maintained. It is unlikely that they would be the mere consequence of cellular adaptations to axial gradients in the pericellular microenvironment, resulting from the processing of ultrafiltrate by the tubuloepithelial cells, as such gradients can hardly be sharp. A possible role for CAMs in intraepithelial sorting is further supported by several reports demonstrating that adhesive properties of individual cells are governed by varying combinations of multiple CAMs [10, 11], and that segregation and border formation occurs between cells expressing a different combination of L-CAM and A-CAM, *in vivo* and *in vitro* [9, 11, 46, 47]. Earlier reports have shown that also cell-matrix interactions are involved in segment-specific differentiation of the nephron. In the developing human kidney,

the α2- and α3-subunits of integrins are characteristically expressed in distal tubules and collecting ducts, and the patterns are retained in the adult nephron [48]. In the mouse kidney, the laminin A chain is found in the basement membrane of proximal tubules only [49].

It thus appears that A-CAM and L-CAM expression are basic properties of cells from proximal and distal tubular lineage, respectively, especially since their expression becomes specifically associated very early with those parts of the S-shaped bodies that will further develop into the proximal and distal tubule, and also since human kidney cells maintain their typical CAM expression *in vitro*, in spite of the fact that some other differentiated properties are significantly less well-preserved [17]. This implies that A-CAM and L-CAM are apt to play key roles in steering segment-specific epithelial regeneration and differentiation after tubular injury. The involvement of several CAMs in regenerative processes has been demonstrated in other organ systems, such as L1 and N-CAM being involved in supporting axon regrowth on the surface of Schwann and fibroblast-like cells during nerve regeneration [50], and an increase in the amount of N-CAM immunoreactivity has been demonstrated in the sarcolemma and intercalated discs of hypertrophic myocardium of the right ventricle of rats [51]. As for the possible occurrence and pathophysiologic consequences of abberant CAM expression in the kidney, there is at present very little information on this matter. Rocco et al [52] reported an early reduction in the mRNA levels encoding N-CAM and E-cadherin in polycystic kidney disease of the mouse, and they deduced that these alterations may contribute to the pathogenesis of cyst formation.

In conclusion, the fetal and adult human kidney expresses the cell-adhesion molecules N-CAM, A-CAM, and L-CAM during specific developmental stages and at specific sites of the nephron. Since cells expressing different combinations of CAMs are known to have reduced adhesive properties, expression of different CAMs along the fetal and adult nephron probably plays a role in establishing and conserving its division into well-delineated morphologically and functionally distinct segments.

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