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The plasma membrane-actin linking protein, ezrin, is a glomerular epithelial cell marker in glomerulogenesis, in the adult kidney and in glomerular injury

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The plasma membrane-actin linking protein, ezrin, is a glomerular epithelial cell marker in glomerulogenesis, in the adult kidney and in glomerular injury.

Background. Ezrin belongs to a family of plasma membrane-cytoskeleton linking, actin binding proteins (Ezrin-radixin-Moesin family) involved in signal transduction, growth control, cell-cell adhesion, and microvilli formation.

Methods. The expression of ezrin was examined in glomerular cells in culture, during kidney development, in the mature kidney, and in five different experimental kidney disease models in the rat.

Results. Ezrin was specifically expressed in glomerular epithelial cells in developing glomeruli in mature glomeruli and in glomerular epithelial cells in culture. Distinct from its other family members, moesin and radixin, which are predominantly expressed in glomerular endothelial and mesangial areas, ezrin protein (by immunohistochemistry) was specifically and exclusively modulated during podocyte injury and regeneration. Ezrin immunohistochemistry was able to visualize cell body attenuation, pseudocysts, and in particular vacuolation of injured podocytes, a feature that usually has to be identified at the ultrastructural level, and was strikingly increased in binucleated podocytes or podocytes that were partially or completely detached from the underlying GBM (frequently also binucleated). Infiltrating macrophages also express ezrin, but can easily be differentiated from podocytes by their round shape and higher level of expression.

Conclusions. Ezrin likely has a role in the cytoskeletal organization, such as reassembling of actin filaments accompanying podocyte injury and regeneration. Since suitable light microscopic markers for the identification of glomerular epithelial cells are rare, ezrin may also be a useful marker for podocytes in normal and injured glomeruli.

The podocyte is a highly differentiated and specialized glomerular cell covering the outer surface of the glomerular basement membrane (GBM), and forms interdigitating foot processes with slit diaphragms as the last layer of the glomerular filtration barrier. Beside its role in forming the glomerular ultrafiltrate, podocytes are thought to counteract the hydrostatic pressure in glomerular capillaries [1] and contribute to the initiation and propagation of glomerular damage, proteinuria, and sclerosis in disease [2–6]. The specialized features of podocytes require a specific cytoskeletal organization. Although an extensive network of microtubules, microfilaments and intermediate filaments containing several contractile components, such as actin, myosin, and α-actin, has been found in podocytes [7], little is known about the cytoskeleton of podocytes in physiologic and pathologic states.

Recently, we have studied the expression of the plasma membrane-cytoskeleton linking proteins, moesin and radixin, in glomerular disease [8]. These actin binding proteins were expressed at elevated levels [in response to platelet-derived growth factor (PDGF), but not basic fibroblast growth factor (bFGF)] in activated/migrating mesangial cells (MC) predominantly in their filopodial cell extensions in experimental mesangial proliferative glomerulonephritis (anti-Thy1 model). Another member of this family of proteins is ezrin, which displays about 75% identity with moesin and radixin at the amino acid level [9]. Ezrin, like moesin and radixin, interacts with the actin cytoskeleton and the plasma membrane at specific cellular locations [10] and is involved in signal transduction and growth control [9]. Ezrin is the target of various protein kinases and phosphorylation of ezrin correlated with membrane ruffling activity in A431 cells after EGF-stimulation [11] or relocalization of ezrin in the microvilli of gastric parietal epithelial cells [12]. This family of proteins is thought to be critical for cell-cell adhesion and microvilli formation [13] and has been characteristically found in very dynamic structures that undergo changes in cell shape [9].

Key words: cytoskeleton, epithelial cell, actin binding protein, kidney development, podocyte injury, injured glomeruli.

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While structural and functional (*in vitro*) similarities between the members of this family are striking, the different tissue distribution of ezrin (primarily epithelial cells) and moesin (primarily endothelial and a restricted set of epithelial cells [14]) implies that these proteins may have distinct functions uniquely adapted to the specific cell types.

In this study we examined the expression of ezrin in cultured glomerular cells, during kidney development, in the adult kidney, and in glomerular disease. We report that ezrin is an excellent and specific marker of undifferentiated and differentiated glomerular epithelial cells during glomerulogenesis, in the adult glomerulus, and in all experimental kidney disease models studied. Ezrin protein (as assessed by immunohistochemistry) was modulated in podocytes during injury induced by different disease mechanisms, and was most strikingly increased in binucleated podocytes or podocytes that were partially or completely detached from the underlying GBM. Ezrin is a useful light microscopic marker of glomerular epithelial cells and may be an important actin binding protein involved in the organization of the complex architecture of the podocyte cytoskeleton in physiologic and pathophysiologic states.

METHODS

Experimental design

Expression of ezrin was analyzed in cultured rat MC, glomerular epithelial cells (GEC), during renal development, and in various glomerular disease models, including a mesangial proliferative glomerulonephritis model (anti-Thy1 disease), experimental membranous nephropathy (passive Heymann nephritis, PHN), aminonucleoside nephritis (PAN), heterologous anti-GBM disease, and glomerulosclerosis induced by 5/6 nephrectomy (remnant kidney model). The expression pattern of ezrin was compared to moesin and radixin. Ezrin protein expression was studied by immunostaining in: (*a*) fetal rats (days 18 and 20 of gestation, $N = 6$ each) and newborn rats, $N = 6$; (*b*) normal adult rats ($N = 6$); and (*c*) at various time points ($N = 3$ to 6) after induction of experimental kidney disease. Glomerular expression of ezrin was graded semiquantitatively in a blinded fashion using a score from 0 to 3 in the different disease models. In order to identify the cells expressing ezrin, tissue was double stained for ezrin [15] and specific cell-type markers for macrophages (ED-1), glomerular endothelial cells (GEN; such as RECA-1 [16]), mesangial cells (MC; such as OX-7) and activated MC (α-sm actin [17]), and a marker for GEC (Wilm's tumor antigen, WT-1) [18]. In addition to immunostaining, the expression of ezrin mRNA was studied by Northern blot analysis, and by *in situ* hybridization using antisense ^{33}P-labeled cRNA probes.

Glomerular cell culture

Rat MC and GEC were prepared from normal glomeruli, characterized and maintained as described previously [19].

Kidney development

Kidneys from 6 to 10 (each) rat embryos day 18 and 20 as well as newborn rats were used for this study.

In vivo disease models

Experimental mesangial proliferative nephritis (anti-Thy1 model) was induced in 180 to 220 g male Wistar rats (Simonsen Laboratories, Gilroy, CA, USA) by intravenous injection of goat anti-rat thymocyte plasma (0.4 cc/100 g body wt) [20]. Rats were sacrificed and renal biopsies were obtained on days 3, 5, 7, 10, and 14 ($N = 6$ to 9 per group). Six normal rats (day 0) served as controls. Passive Heymann nephritis, a model of membranous nephropathy, was induced in male Sprague-Dawley rats (200 to 230 g body wt, $N = 6$; Simonsen Laboratories) by a single intraperitoneal injection (0.5 ml/100 g) of sheep anti-Fx1A antibody (prepared as described elsewhere) [20]. Control rats were injected with normal sheep serum. Twenty-four hour proteinuria was assessed in all rats before sacrifice to assure significant disease. Rats were sacrificed on days 5, 10, 15, 21, and 30 and renal biopsies were obtained. Aminonucleoside nephrosis, a model of minimal change disease, was induced in male Sprague-Dawley rats (200 to 230 g body wt, $N = 6$; Simonsen Laboratories) by a single intravenous injection of puromycin (0.1 mg/kg; ICN Pharmaceuticals, Cleveland, OH, USA) [20]. Rats injected with 0.9% NaCl served as controls ($N = 6$). Twenty-four hour proteinuria was assessed in all rats before sacrifice to assure significant disease. Rats were sacrificed on days 5, 11, 14, and 21 and renal biopsies were obtained. Anti-GBM nephritis was induced in three rats each by a single intravenous injection of sheep anti-rat GBM antibody as described previously [21] and renal biopsies were obtained on days 0, 1, and 5. A model of chronic renal failure accompanied by high blood pressure, 5/6 nephrectomy, was induced in male Sprague-Dawley rats (Simonsen Laboratories, 140 to 160 g body wt at the start of the experiment, $N = 3$ to 6) by a right subcapsular nephrectomy and an infarction of two thirds of the left kidney via ligation of the posterior and one or two anterior extrarenal branches of the main renal artery [22]. Sham operated rats served as controls. Animals were sacrificed at 1, 2, 3, 4, 7.5, and 10 weeks.

Immunohistochemistry

Renal biopsies were fixed in methyl Carnoy's solution for immunohistochemistry or 10% formalin for *in situ* hybridization studies and embedded in paraffin [17]. Indirect immunoperoxidase and immunofluorescent staining of 4 μm sections was performed as described previously [17]

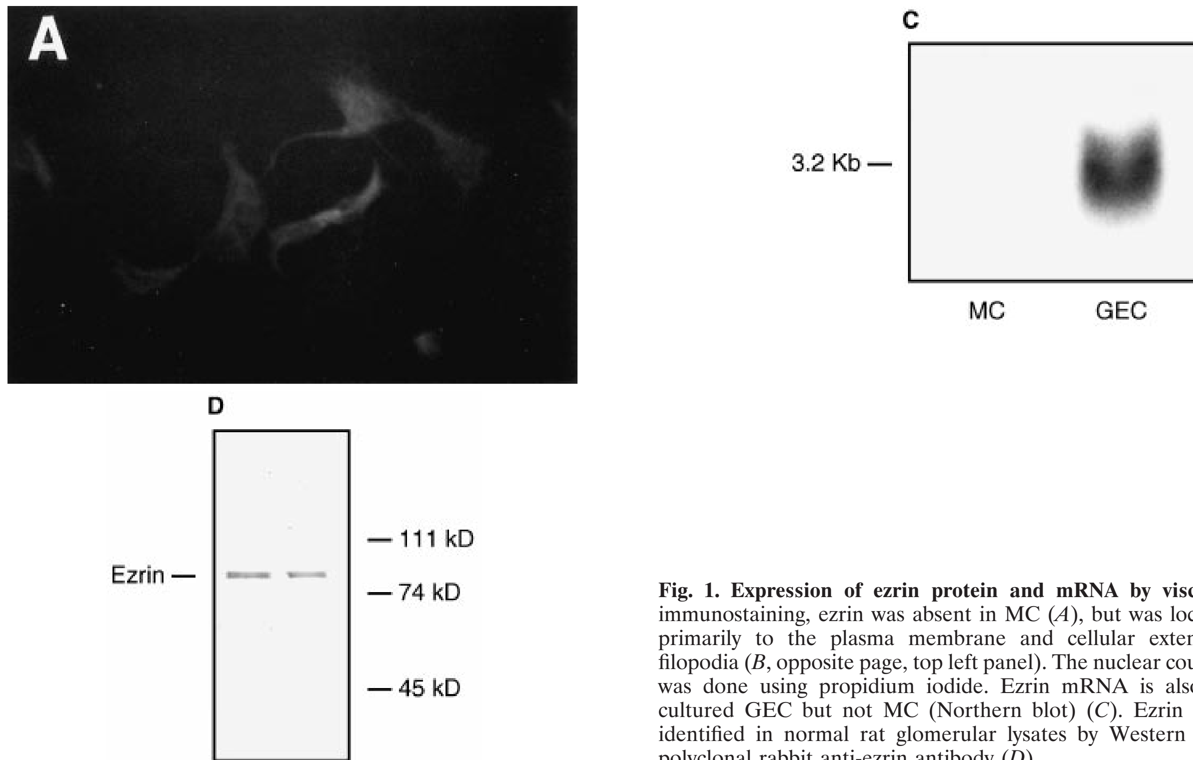


Fig. 1. Expression of ezrin protein and mRNA by visceral GEC. By immunostaining, ezrin was absent in MC (*A*), but was localized in GEC primarily to the plasma membrane and cellular extensions such as filopodia (*B*, opposite page, top left panel). The nuclear counterstain (red) was done using propidium iodide. Ezrin mRNA is also expressed in cultured GEC but not MC (Northern blot) (*C*). Ezrin protein is also identified in normal rat glomerular lysates by Western blot using the polyclonal rabbit anti-ezrin antibody (*D*).

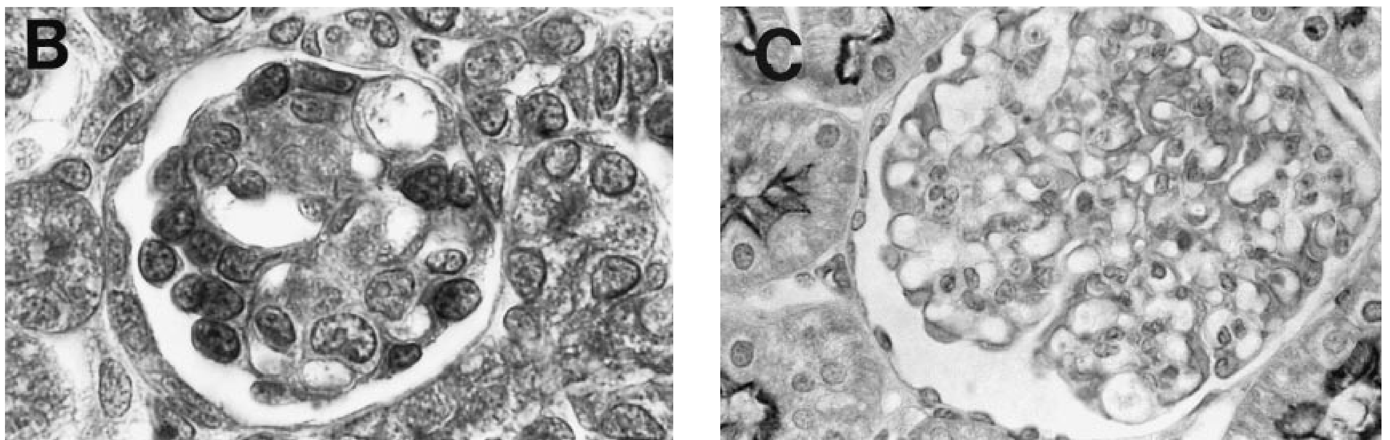
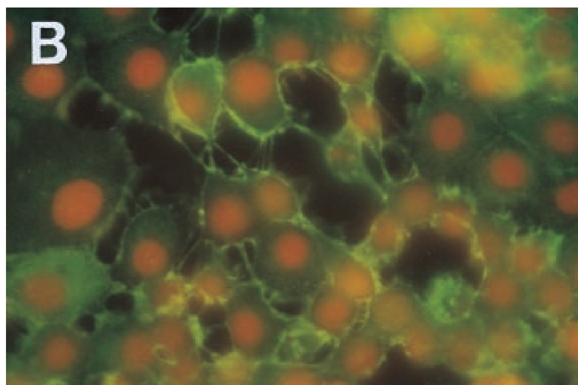


Fig. 2. Expression of ezrin in rat kidney development and in the adult kidney. In glomeruli from newborn rats, the double immunofluorescent staining for ezrin (FITC-green) and collagen IV (TRIC-red, for GBM and MC) on ultrathin sections reveals the specific parietal and visceral epithelial cell expression of ezrin in the developing glomerulus and in proximal tubular cells (*A*, opposite page, here a day 20 rat embryo). Abbreviations are: bv, blood vessel; ms, mesangium; Bc, Bowman's capsule; pt, proximal tubule. The epithelial specificity for ezrin was confirmed by double staining for ezrin (brown) and WT-1 (black nuclear staining) (*B*, above). In the adult rat glomerulus, ezrin protein is expressed by visceral and parietal GEC and by proximal tubular cells (*C*, above). Double immunostaining for ezrin (FITC-green) and collagen IV (TRIC-red, stains GBM and mesangium) on ultrathin sections (*D*, opposite page) demonstrates the exclusive expression of ezrin by podocytes seen at the outer side of the GBM (marked by collagen IV in red). The capillary lumen is marked by asterisks.

using a specific polyclonal rabbit anti-ezrin antibody (pAbE) [15]. In addition, tissue was stained with a polyclonal rabbit anti-moesin/radixin antibody (pAbMoR) that specifically recognizes radixin and moesin or an affinity purified anti moesin antibody (pAbMo) that is specific for moesin, but not ezrin or radixin as described previously [8, 15], with a murine IgG_1 mAb, ED-1, to a cytoplasmic antigen present in monocytes, macrophages and dendritic cells (Bioproducts for Science, Inc., Indianapolis, IN, USA), a murine IgG_1 mAb against the endothelial antigen RECA-1 [16], an affinity-purified rabbit polyclonal Ab against Wilm's tumor (WT-1) antigen (Santa Cruz Biotech, Heidelberg, Germany [18]), a murine IgG1 mAb against the Thy1 antigen on MC (OX-7; Accurate Chemical Corp.,



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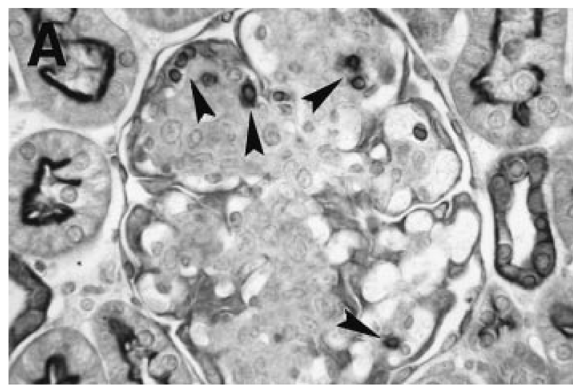


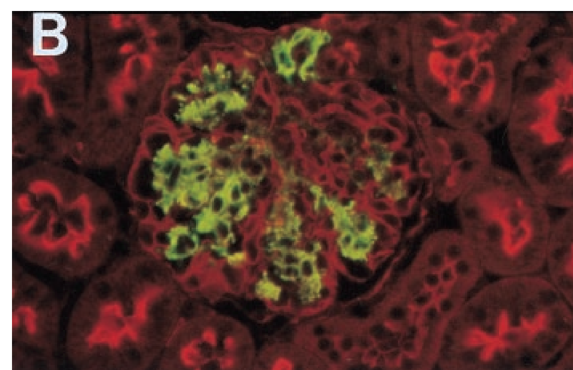
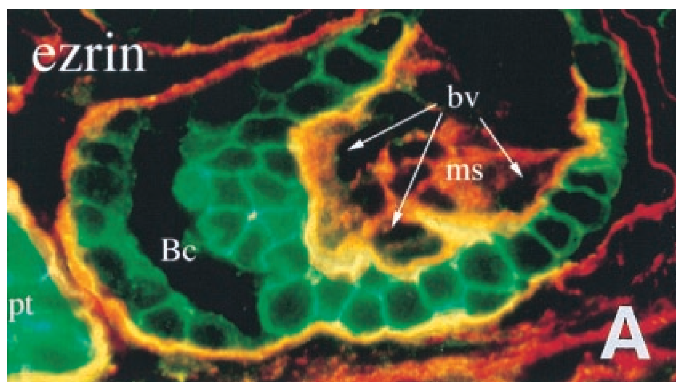


Fig. 4. Expression of ezrin (by immunostaining) in mesangial cell injury. In mesangial proliferative glomerulonephritis (*A and B*, here on day 5) ezrin protein is constitutively expressed by GEC and also by infiltrating mononuclear cells (A, arrows) that were shown to be ED-1 positive macrophages (not shown here). Double immunofluorescent staining (B) for ezrin in red (Texas red) and alpha sm-actin (a marker of activated MC) in green demonstrates the non-overlapping distribution of these proteins during MC injury. A double-labeled cell expressing both ezrin (red) and α-sm actin (green) would appear orange on the photograph.



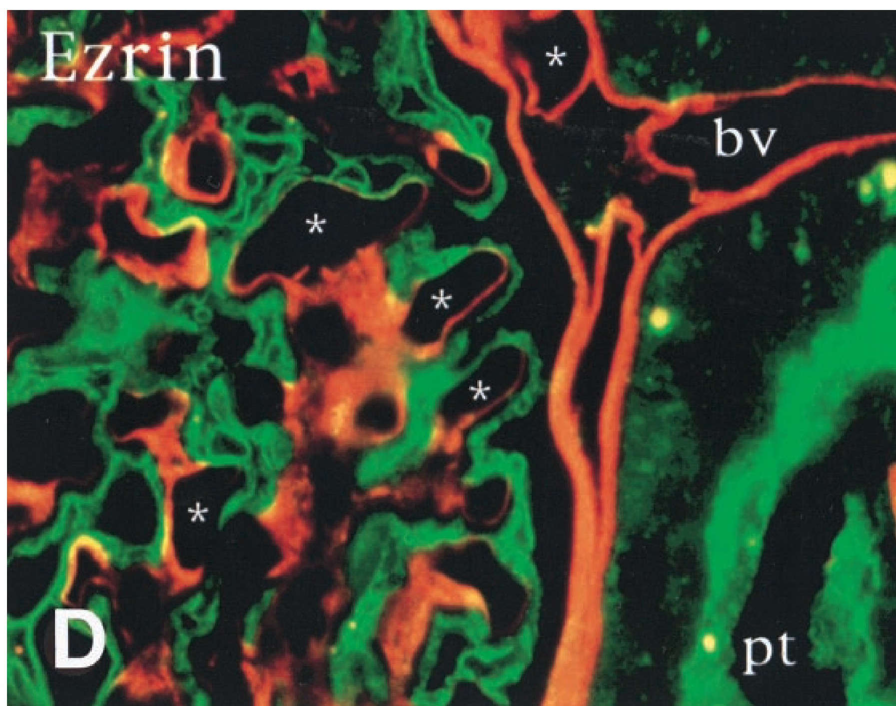


Fig. 2, A and D (left).

Westbury, NY, USA), a murine IgG_2 mAb against α-smooth muscle actin [23] (Sigma Chemical Co, St. Louis, MO, USA), and a affinity-purified polyclonal goat anti-human/bovine type IV collagen antibody (Southern Biotechnology, Birmingham, AL, USA). Negative controls for immunostaining included either omitting the primary antibody or substitution of the primary antibody with equivalent concentrations of an irrelevant murine monoclonal antibody or preimmune rabbit IgG. For each biopsy, 20 to 50 glomerular cross sections were evaluated in a blinded fashion. Glomerular expression of ezrin was graded semiquantitatively [8] and reflected changes in the intensity of staining: 0 = absent staining; 1+ = weak, but identifiable staining; 2+ = intermediate, clearly increased staining; 3+ = dramatically increased, strong staining.

Immunohistochemical double staining

To determine the cell types expressing ezrin, double immunostaining with an ezrin-specific antibody and cell specific markers was performed using an indirect immunofluorescent and/or immunoperoxidase technique [8, 24]. The specific polyclonal ezrin-antibody was incubated overnight at 4°C, followed by Texas red-or peroxidase-conjugated goat anti-rabbit IgG for one hour at reverse transcription (RT). Color development for the peroxidase staining was done using diaminobenzidine (DAB) without nickel chloride (NiCl, brown) followed by incubation in 3% H_2O_2 in methanol for 20 minutes to prevent any remaining peroxidase activity prior to application of the second primary antibody. Afterwards, cell specific antibodies for glomerular MC (OX-7), GEN (RECA-1), GBM (collagen IV) or epithelial cells (WT-1) were applied overnight at 4°C, followed by biotinylated rabbit anti-mouse IgG1 (Zymed, San Francisco, CA, USA), and FITC- or peroxidase-conjugated streptavidin D [24]. Color development for the second antibody was done using DAB with NiCl (black) or True blue (blue; Kirgaard & Perry Laboratories, Gaithersburg, MD, USA). To determine whether activated MCs or infiltrating macrophages specifically express ezrin, double staining was performed using ezrin specific antibodies with α-sm actin or ED-1 antibodies, respectively, using an equivalent protocol. Controls for all double staining procedures consisted of replacing either one of the primary antibodies with an irrelevant mouse monoclonal antibody or preimmune rabbit IgG.

High resolution immunostaining of ezrin using ultrathin cryosections

To increase resolution of ezrin or moesin immunostaining the following technique was applied to additional 20-day-old rat embryos and adult rats. These rats were sacrificed and their tissues perfused by cannulating the aorta with isotonic Ringers solution until all the blood was removed, followed by perfusion with PLP fixative. The tissues were cut into < 1 mm [3] cubes and post fixed for one hour at 4°C in PLP. The fixed tissues were rinsed with PBS and cryoprotected by infiltration with 2.4 M sucrose. Tissues were kept indefinitely in 2.4 M sucrose at 4°C. 0.5 to 1 μm frozen sections were taken using a Reichard Ultracut E microtome equipped for cryoultramicrotomy. The sections were blocked for 10 minutes with 3%BSA, and stained with primary antibodies for one hour at RT. After washing with PBS, the sections were stained with FITC- or Texas Red-conjugated secondary antibodies, washed and mounted with glycerol and phenylenediamine. The pictures were taken with an Olymusepifluorescence microscope.

Immunoblotting of ezrin and specificity of the antibodies

Immunoblotting was performed as described previously [8] and 5 μg of total glomerular protein was loaded per lane. Amieva and Furthmayr have extensively characterized the antibodies used in this study, as shown previously [15]. The ezrin antiserum (pAbE) reacted specifically with the 82 kD band of ezrin without crossreactivity to moesin or radixin [8, 15]. The immunostaining using pAbE could not be absorbed by adding recombinant moesin and radixin, while staining using pAbMoR for radixin/moesin or pAbMo for moesin only could be specifically abolished adding the recombinant proteins as reported previously [8].

Preparation of total RNA from cultured cells and isolated glomeruli followed by Northern analysis

Total RNA was extracted from cultured MC and GEC or isolated glomeruli (differential sieving method as described previously) with RNAzol B (Cinna/Biotecx Laboratories, Friendswood, TX, USA) followed by LiCl precipitation [17]. Ten micrograms of cultured cell or 15 μg of glomerular RNA per lane were electrophoresed through a 3% formaldehyde, 1% agarose gel and transferred to a nylon membrane (Hybond N+, Amersham) as described previously [4]. A fragment of the pig or human ezrin cDNA clone [25] was isolated, purified with the SpinBind-system (FMC, Rockland, ME, USA), and radiolabeled with [^{32}p] dCTP (10 mCi/ml; New England Nuclear, Boston, MA, USA) by random primer extension. Membranes were prehybridized for 20 minutes, hybridized with 2×10^6 cpm probe/ml for one to two hours at 68°C in Quickhyb solution (Stratagene, La Jolla, CA, USA) and washed with $0.1 \times$ SSPE, 0.1% SDS twice for 30 minutes at 55°C. All Northern blots were repeated at least three times with RNA from different experiments.

In situ hybridization for ezrin mRNA

Ezrin mRNA was detected by *in situ* hybridization on formalin-fixed tissue using ^{32}P-labeled anti-sense and sense-cRNA probes to human ezrin cDNA [25]. Hybridization was performed using a protocol of Wilcox et al [26]. After developing, sections were counterstained with hematoxylin/eosin, dehydrated and coverslipped.

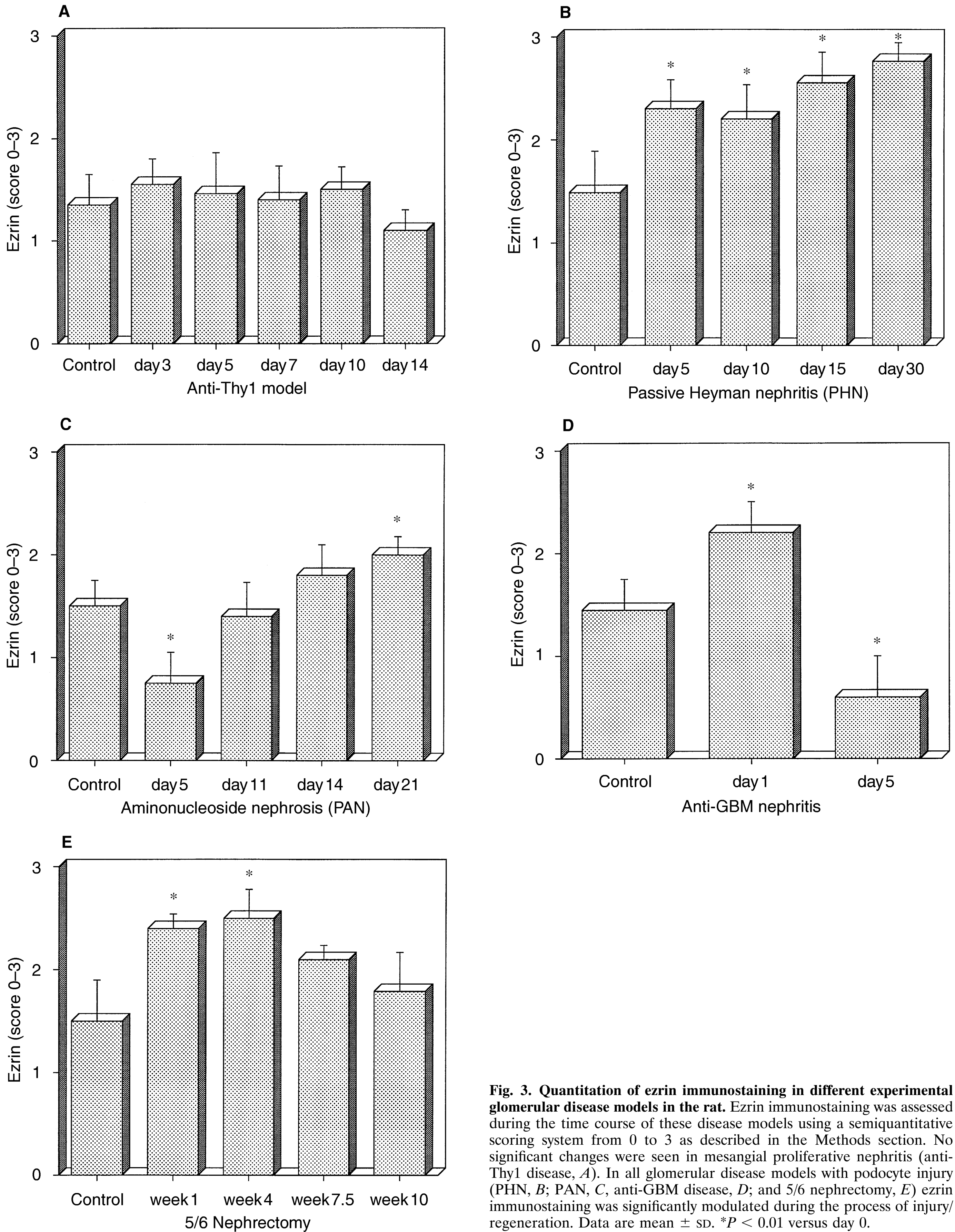


Fig. 3. Quantitation of ezrin immunostaining in different experimental glomerular disease models in the rat. Ezrin immunostaining was assessed during the time course of these disease models using a semiquantitative scoring system from 0 to 3 as described in the Methods section. No significant changes were seen in mesangial proliferative nephritis (anti-Thy1 disease, *A*). In all glomerular disease models with podocyte injury (PHN, *B*; PAN, *C*, anti-GBM disease, *D*; and 5/6 nephrectomy, *E*) ezrin immunostaining was significantly modulated during the process of injury/regeneration. Data are mean ± SD. $^*P < 0.01$ versus day 0.

Table 1. Ezrin protein expression in anti-Thy1 nephritis, aminonucleoside nephrosis (PAN), passive Heymann nephritis (PHN), anti-GBM disease and 5/6 nephrectomy by immunostaining using a semiquantitative visual scoring system (0 to 3)

Days	Anti-Thy1 model	PAN	PHN	Anti-GBM disease	5/6 Nephrectomy
Control	1.35 ± 0.30	1.50 ± 0.25	1.49 ± 0.40	1.45 ± 0.30	1.05 ± 0.40
Day 1/3	1.55 ± 0.25	ND	ND	2.20 ± 0.3[a]	ND
Day 5	1.46 ± 0.40	0.80 ± 0.3[a]	2.30 ± 0.4[a]	0.60 ± 0.4[a]	ND
Day 7	1.28 ± 0.33	ND	ND	ND	2.70 ± 0.1[a]
Day 10/11	1.50 ± 0.22	1.40 ± 0.33	2.20 ± 0.3[a]	ND	ND
Day 14/15	1.10 ± 0.20	1.80 ± 0.30	2.55 ± 0.3[a]	ND	ND
Week 3/4	ND	2.10 ± 0.2[a]	2.76 ± 0.2[a]	ND	2.70 ± 0.3[a]
Week 7.5	ND	ND	ND	ND	2.10 ± 0.24
Week 10	ND	ND	ND	ND	1.85 ± 0.38

Data are mean ± SD, ND means not done.
[a] $P < 0.01$ versus control

Statistical analysis

All values are expressed as mean ± SD. Statistical significance (defined as $P < 0.05$) was evaluated using the Student's t test or one way analysis of variance with modified t test was performed using the Bonferroni method.

RESULTS

Ezrin is a glomerular epithelial cell marker in cultured glomerular cells

Immunohistochemistry, Western blot, and Northern blot analysis demonstrated that ezrin protein and mRNA were endogenously expressed in cultured visceral GEC, and were absent in cultured MC (Fig. 1). GEC staining of ezrin was predominant in plasma membrane and filopodia and there was also faint perinuclear staining (Fig. 1B).

Ezrin is an epithelial cell marker in rat kidney development and in the adult kidney

Immunostaining for ezrin protein in 18, 20-day-old embryonic, and newborn rat kidneys revealed that ezrin is consistently and specifically expressed in the epithelial cell clusters characterizing the early vesicle, comma and S-stages as well as in parietal and visceral epithelial cells of more mature forms during all stages of glomerulogenesis, as demonstrated by double immunostaining for ezrin and collagen IV (characterizing MC and GBM, Fig. 2A) or by double staining for ezrin and WT-1 (another GEC marker; Fig. 2B). In addition to its epithelial expression pattern during glomerulogenesis, ezrin is also expressed in ureteric buds, most parts of the tubular system, and the urothelium (not shown). *In situ* hybridization with antisense but not sense cRNA for ezrin confirmed expression of ezrin mRNA in the developing glomeruli (not shown).

In the normal rat glomerulus, ezrin is expressed by parietal and visceral GEC (Fig. 2C). Unlike its other family members, moesin and radixin [8], ezrin could not be detected in GEN or MC. Double immunostaining for ezrin and collagen IV (stains GBM and MC) on ultrathin sections clearly demonstrated the exclusive expression of ezrin on the outer (podocytic) side of the GBM (marked by collagen IV; Fig. 2D). Double staining with an antibody against WT-1, which labels GEC in the rat glomerulus [18], demonstrated colocalization of both proteins (not shown). The GEC staining pattern for ezrin was also confirmed by double labeling with cell specific markers for GEN or MC, using the antibodies RECA-1 or OX-7, respectively. Ezrin is also expressed in collecting ducts, the urothelium, in the brush border microvilli on the apical side of proximal tubular epithelial cells, and in a subset of distal tubules on the basolateral side (not shown).

Ezrin is a glomerular epithelial cell marker in experimental glomerular disease

While ezrin is expressed solely by glomerular epithelial cells in glomeruli in development and in the adult, it could still be induced by MC and/or GEN in diseased states. Therefore, we examined ezrin expression in several glomerular disease models in which different glomerular cell types are injured.

Ezrin is not expressed by MC during experimental mesangial proliferative glomerulonephritis (anti-Thy1 model). In anti-Thy1 disease, a model of mesangial proliferative glomerulonephritis with predominant MC injury, ezrin protein expression by podocytes did not change at any time point studied (by immunostaining, Figs. 3A, 4A, Table 1; or by *in situ* hybridization, not shown). During the time course of disease, ezrin protein was always expressed by GEC, but not by regenerating and proliferating MC, as demonstrated by double staining for ezrin and α-sm-actin (a marker of activated MC; Fig. 4B). Infiltrating mononuclear cells also stained strongly positive for ezrin (Fig. 4A) and were identified as ED-1 positive macrophages (by double immunostaining, not shown).

Ezrin is modulated during podocyte injury. Ezrin expression was also studied in glomerular disease models in which injury to the podocyte occurs. In experimental membranous glomerulopathy (PHN), where podocyte injury is complement-mediated, ezrin immunostaining was focally increased in podocytes on day 5, and increased in a homogenous pattern up to day 30 (Fig. 3B). Strong ezrin

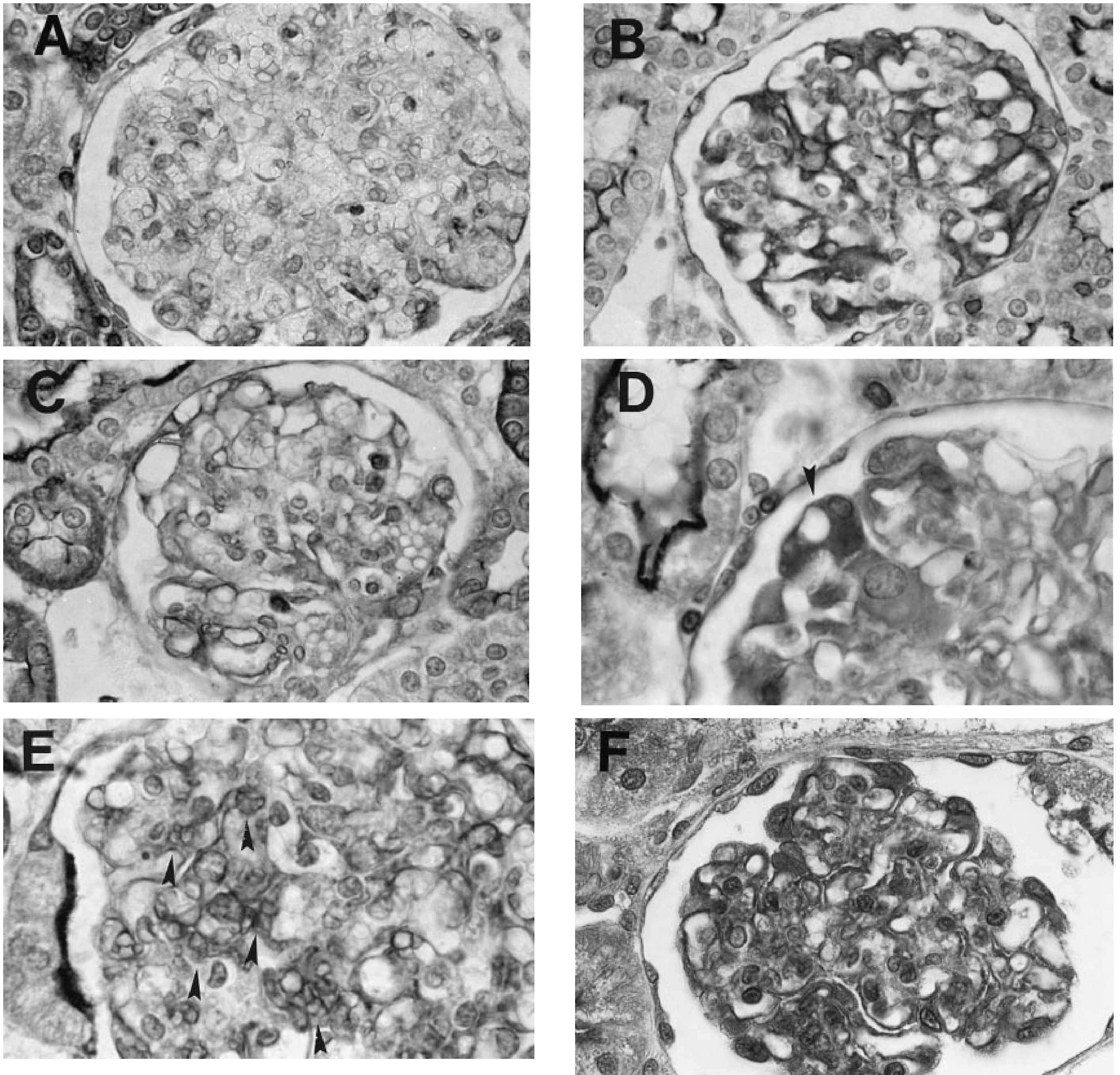


Fig. 5. Expression of ezrin in models with podocyte injury. Ezrin expression (black) is focally decreased in podocytes in aminonucleoside nephrosis (*A*, PAN day 5). This characteristic inhomogenous pattern of ezrin was gradually replaced by a generalized increase in ezrin expression in podocytes by day 21 of disease (*B*, PAN day 21). Ezrin immunostaining identified features of podocytic injury such as adhesion with parietal GEC (*C*, PAN day 14), pseudocyst formation (*D*, PAN day 14, arrowhead), and podocyte vacuolation (*E*, PAN day 5, arrowheads) that was maximal between days 5 and 11 of disease and quite characteristic for PAN. Ezrin (brown) was also shown to colocalize with another GEC marker, WT-1 (black nuclear stain) in podocytes in aminonucleoside nephrosis (day 11; *F*).

immunostaining was especially apparent in binucleated podocytes (peak on day 10; Fig. 6).

In the aminonucleoside model, ezrin immunostaining was focally decreased on day 5 (Figs. 3C and 5A). Later in the disease (days 11 to 14), focal increases of ezrin immunostaining could be observed in some podocytes, whereas in other podocytes in the same glomerulus ezrin expression was still diminished. This characteristic inhomogenous pattern of ezrin was gradually replaced by a generalized increase in ezrin expression in podocytes by day 21 of the disease (Fig. 5B). Ezrin immunostaining identified areas of podocytic injury such as adhesion with parietal GEC (Fig.

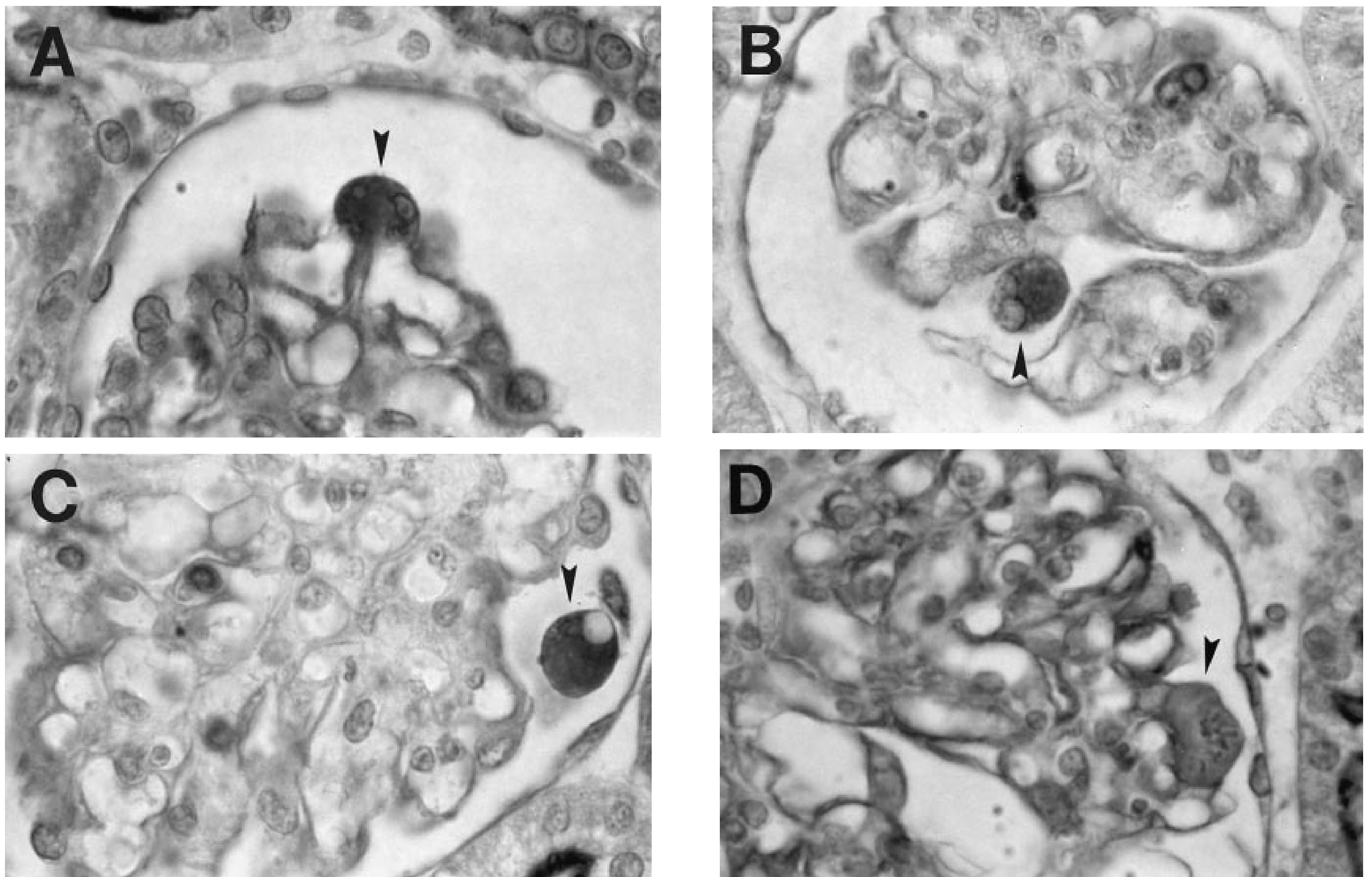


Fig. 6. Ezrin staining identifies mitotic, polynucleic and detached podocytes. The highest levels of ezrin protein expression were observed in polynucleated podocytes (*A*, anti-GBM disease day 5) as well as in injured podocytes that were either partially (*B*, anti-GBM disease day 5) or completely detached from the GBM (*C*, anti-GBM disease day 1). Ezrin was also increased in mitotic podocytes (*D*, PHN day 10).

5C), pseudocyst formation (Fig. 5D), and podocyte vacuolation (Fig. 5E), which were maximal between days 5 and 11 of disease. Strong ezrin immunostaining was apparent in polynucleated podocytes on day 11. Moesin/radixin expression in both models with podocyte injury (PHN, PAN) was restricted to endothelial/mesangial areas and did not change substantially in these disease models (data not shown).

In anti-GBM disease, ezrin was strongly expressed in podocytes on day 1, but rarely detectable on day 5 of disease (Fig. 3D). Despite the decreased expression in most of the podocytes on day 5, ezrin was highly expressed in podocytes that were either polynucleic (Fig. 6A) or completely (or almost completely) detached from the GBM (Fig. 6B,C).

In the 5/6 nephrectomy model where combined endothelial, mesangial, and glomerular epithelial cell injury is accompanied by systemic and glomerular hypertension and leads to a remodeling of the glomerular architecture, ezrin expression was again specific for GEC and transiently as well as focally increased early (week 1 and 2) and was decreased late (weeks 7.5 and 10; Fig. 3E). Despite an overall decrease late in this model, ezrin immunostaining was increased in areas of adhesions between parietal GEC and the glomerular tuft with denuded GBM or injured podocytes. At sites of synechia formation, a dramatic invasion and/or proliferation of ezrin-positive parietal GEC could be shown.

Double immunostaining for ezrin and WT-1 (a GEC marker) in these disease models also demonstrated the podocyte-specific expression of ezrin protein (Fig. 5F).

The specific modulation of ezrin protein by podocytes in different glomerular disease models apparent by immunostaining was not seen at the mRNA level. Since ezrin mRNA could not be detected by Northern blot analysis using 15 μg total RNA per lane prepared from isolated glomeruli, radioactive *in situ* hybridization was done and revealed a constitutive low level expression of ezrin mRNA in normal glomeruli that did not show any substantial change in glomeruli, even in disease models where ezrin expression was clearly modulated at the protein level by immunostaining (PAN, PHN, 5/6 nephrectomy; data not shown). Controls using sense cRNA to ezrin were always negative.

Ezrin is increased in mitotic and polynucleic podocytes as well as in podocytes that were partially or completely detached from the underlying GBM

In addition to a specific modulation of podocytes *in vivo* during injury, the intensity of ezrin immunostaining was most obviously increased in polynucleated podocytes (Fig. 6). Polynucleated podocytes could be found in PAN, PHN, and anti-GBM disease and consistently showed strongest expression of ezrin (by immunohistochemistry, Fig. 6). The strong intensity of ezrin immunostaining in polynucleated podocytes compared to the other glomerular cells allowed easy identification of these cells in tissue sections. Frequently, polynucleated podocytes were partially or completely detached from the underlying GBM and changed into big round ezrin positive cells without the typical appearance of podocytes. Interestingly, mitotic podocytes were also strongly positive for ezrin (Fig. 6D).

DISCUSSION

Podocytes are specialized glomerular cells important in the maintenance of the filtration barrier, as well as in the support of the capillary tuft, GBM turnover and immunologic functions [6]. One of the unique features of podocytes are their interdigitating foot processes that are thought to be critical in providing tensile support to the GBM [1]. They exhibit a prominent actin cytoskeleton, but in spite of this importance, little is known about how these structures form, how they are maintained, and how the cytoskeleton influences this and other functions.

In this article, we report that the cytoskeleton-linking protein, ezrin, is constitutively expressed in GEC. Unlike the related proteins moesin and radixin, which are expressed by mesangial cells and endothelial cells [8], ezrin is expressed only by visceral and parietal GEC in glomeruli. Ezrin expression (mRNA and protein) could be demonstrated in GEC in culture, during glomerulogenesis, and in the adult kidney.

Although ezrin expression was specific for visceral and parietal GEC within the glomerulus, the intensity of the immunostaining varied in different glomerular disease models. Thus, a relative decrease in ezrin immunostaining was observed in GEC in the aminonucleoside (day 5), the anti-GBM (day 5) and in the remnant kidney (weeks 7.5 and 10) models. This phenomenon presumably reflects cytotoxicity to the GEC. In contrast, an increased staining intensity for ezrin in GEC was noted in the passive Heymann model (days 5 to 30), during the early phase of the anti-GBM model (day 1), and in the remnant kidney model (weeks 2 to 3). In none of these instances, however, did we detect significant changes in ezrin mRNA levels.

One interesting finding was the strong expression of ezrin in a subset of podocytes undergoing injury and/or proliferation. Podocyte injury leads to a stereotypic structural change that includes foot process effacement, vacuolization, hypertrophy, cell body attenuation, pseudocyst formation, and detachment from the GBM [27]. These characteristic maladaptive changes in podocytes have been observed ultrastructurally in various animal models [28–30] and in human disease [31] and have been postulated to play a major role in the pathogenesis of progressive glomerulosclerosis [2]. Identification of these pathologic findings has usually required ultrastructural (electron microscopy) studies. However, by using anti-ezrin antibodies, we were able to identify changes in the podocyte population at the light microscopic level, such as vacuolization, the appearance of polynucleic cells, or of cells involved in synechiae formation, detached cells, and even mitotic podocytes. This suggests that immunostaining for ezrin may be a sensitive way of monitoring for GEC injury.

The functions of ezrin are not fully understood. *In vitro* studies have implicated ezrin and related cytoskeleton-linking proteins in microvilli formations, cell adhesion, migration and growth [9, 13]. Cosedimentation experiments have demonstrated that ezrin preferentially interacts with cytoplasmic β-, rather than α-actin [32]. This suggests ezrin may be involved in the modulation of the GEC shape, contraction, or attachment to the GBM, all actions that may be critical for GEC function. In this context, the observation that ezrin expression was highest in mitotic, binucleated or polynucleic podocytes and in podocytes completely or nearly detached to the GBM may reflect the need to adapt to mechanical, toxic or immunological injury. If adaptation fails, podocytes may become completely detached, round up and possibly die (by apoptosis or necrosis). This pathway may be of relevance to glomerular disease, since loss of podocytes is believed to predispose to progressive scarring [2].

In conclusion, the plasma membrane-cytoskeleton linking protein, ezrin, is a GEC marker during glomerulogenesis, in the mature kidney and during glomerular injury. Ezrin protein is modulated during podocyte injury and regeneration in different experimental models. Immunostaining of ezrin is considerably enhanced in polynucleated podocytes or in podocytes that are detached from the underlying GBM, and this allows for the identification of features of injured podocytes such as vacuolization by light microscopy. Ezrin immunohistochemistry is a useful tool for studying GEC (patho)physiology, and ezrin may be an important structural and/or signaling protein in mediating cytoskeletal arrangements during GEC injury.

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APPENDIX

Abbreviations used in this article are: Ang II, angiotensin II; ATS, anti-thymocyte serum (plasma); BCIP/NBT, 5-Bromo-4-Chloro-3-Indolyl Phosphate; bFGF, basic fibroblast growth factor; cpm, counts per minute; DAB, diaminobenzidine; ECM, extracellular matrix; EGF, epidermal growth factor; FCS, fetal calf serum; GBM, glomerular basement membrane; GEC, glomerular epithelial cell; GEN, glomerular endothelial cell; ISH, *in situ* hybridization; MC, mesangial cell; PAN, puromycin aminonucleoside nephrosis; PCNA, proliferating cell nuclear antigen; PDGF, platelet-derived growth factor; PHN, passive Heyman nephritis; sm, smooth muscle; SSPE, standard saline phosphate EDTA; SSC, standard saline citrate.

REFERENCES

1. Kriz W, Hackenthal E, Nobiling R, Sakai T, Elger M: A role for podocytes to counteract capillarywall distension. *Kidney Int* 45:369–376, 1994
2. Kriz W, Elger M, Nagata M, Kretzler M, Uiker S, Koeppen-Hagemann I, Tenschert S, Lemley KV: The role of podocytes in the development of glomerular sclerosis. *Kidney Int* 45:64–72, 1994
3. Couser WG, Johnson RJ: Mechanisms of progressive renal disease in glomerulonephritis. *Am J Kidney Dis* 23:193–8, 1994
4. Rennke HG: How does glomerular epithelial cell injury contribute to progressive glomerular damage? *Kidney Int* 45:58–63, 1994
5. Kerjaschki D: Dysfunctions of cell biological mechenisms of visceral epithelial cell (podocytes) in glomerular diseases. *Kidney Int* 45:300–313, 1994
6. Adler S: Glomerular epithelial cells (chapt 31), in *Immunologic Renal Diseases*, edited by Neilson EG, Couser WG, Philadelphia, Lippincott-Raven Publishers, 1997, pp 1–13
7. Drenckhahn D, Franke R-P: Ultrastructural organization of contractile and cytoskeletal proteins in glomerular podocytes of chicken, rat, and man. *Lab Invest* 59:673–682, 1988
8. Hugo C, Hugo C, Pichler R, Gordon K, Schmidt R, Amieva M, Couser W, Johnson R: The cytoskeletal linking proteins, moesin and radixin, are upregulated by platelet-derived growth factor, but not basic fibroblast growth factor in experimental mesangial proliferative glomerulonephritis. *J Clin Invest* 97:2499–2508, 1996
9. Arpin M, Algrain M, Louvard D: Membrane-actin microfilament connections: An increasing diversity of players related to band 4.1. *Curr Opin Cell Biol* 6:136–141, 1994
10. Turunen O, Wahlstrom T, Vaheri A: Ezrin has a COOH-terminal actin-binding site that is conserved in the ezrin protein family. *J Cell Biol* 126:1445–1453, 1994
11. Bretscher A: Rapid phosphorylation and reorganization of ezrin and spectrin accompany morphological changes induced in A-431 cells by epidermal growth factor. *J Cell Biol* 108:921–930, 1989
12. Hanzel D, Reggio H, Bretscher A, Forte JG, Mangeat P: The secretion-stimulated 80K phosphoprotein of parietal cells is ezrin, and has properties of a membrane cytoskeletal linker in the induced apical microvilli. *EMBO J* 10:2363–73, 1991 [published erratum appears in *EMBO J* 10:3978–3981, 1991]
13. Takeuchi K, Sato N, Kasahara H, Funayama N, Nagafuchi A, Yonemura S, Tsukita S, Tsukita S: Perturbation of cell adhesion and microvilli formation by antisense oligonucleotides to ERM family members. *J Cell Biol* 125:1371–1384, 1994
14. Berryman M, Franck Z, Bretscher A: Ezrin is concentrated in the apical microvilli of a wide variety of epithelial cells whereas moesin is found primarily in endothelial cells. *J Cell Sci* 105:1025–1043, 1993
15. Amieva MR, Furthmayr H: Subcellular localization of moesin in dynamic filopodia, retraction fibers and other structures involved in substrate exploration, attachment and cell-cell contacts. *Exp Cell Res* 219:180–196, 1995
16. Duijvestijn AM, van Goor H, Klatter F, Majoor GD, van Bussel E, van Breda Vriesman PJ: Antibodies defining rat endothelial cells: Reca-1, a pan-endothelial cell-specific monoclonal antibody. *Lab Invest* 66:459–466, 1992
17. Johnson RJ, Iida H, Alpers CE, Majesky MW, Schwartz SM, Pritzl P, Gordon K, Gown AM: Expression of smooth muscle cell phenotype by rat mesangial cells in immune complex nephritis. Alpha-smooth muscle actin is a marker of mesangial cell proliferation. *J Clin Invest* 87:847–858, 1991
18. Mundlos S, Pelletier J, Darveau A, Bachmann M, Winterpracht A, Zabel R: Nuclear localization of the protein encoded by the Wilm's tumor gene WT1 in embryonic and adult tissues. *Development* 119:1329–1341, 1993
19. Adler S, Baker PJ, Johnson RJ, Ochi RF, Pritzl P, Couser WG: Complement membrane attack complex stimulates production of reactive oxygen metabolites by cultured rat mesangial cells. *J Clin Invest* 77:762–767, 1986
20. Pichler R, Giachelli CM, Lombardi D, Pippin J, Gordon K, Alpers CE, Schwartz SM, Johnson RJ: Tubulointerstitial disease in glomerulonephritis. Potential role of osteopontin (uropontin). *Am J Pathol* 144:915–926, 1994
21. Couser WG, Darby C, Salant DJ, Adler S, Stilmant MM, Lowenstein LM: Anti-GBM antibody-induced proteinuria in isolated perfused rat kidney. *Am J Physiol* 249:F241–F250, 1985
22. Floege J, Burns MW, Alpers CE, Yoshimura A, Pritzl P, Gordon K, Seifert RA, Bowen PDF, Couser WG, Johnson RJ: Glomerular cell proliferation and PDGF expression precede glomerulosclerosis in the remnant kidney model. *Kidney Int* 41:297–309, 1992
23. Skalli O, Ropraz P, Trzeciak A, Benzonana G, Gillessen D, Gabbiani G: A monoclonal antibody against alpha-smooth muscle actin: A new probe for smooth muscle cell differentiation. *J Cell Biol* 103:2787–2796, 1986
24. Hugo C, Pichler R, Meek R, Gordon K, Kyriakides T, Floege J, Bornstein P, Couser W, Johnson RJ: Thrombospondin 1 is expressed by proliferating mesangial cells in vivo and is upregulated by PDGF and bFGF. *Kidney Int* 48:1846–1856, 1995
25. Gould KL, Bretscher A, Esch FS, Hunter T: cDNA cloning and sequencing of the protein-tyrosine kinase substrate, ezrin, reveals homology to band 4.1. *EMBO J* 8:4133–4142, 1991
26. Wilcox JN, Smith KM, Schwartz SM, Gordon D: Localization of tissue factor in the normal vessel wall and in the atherosclerotic plaque. *Proc Natl Acad Sci USA* 86:2839–2843, 1989
27. Mundel P, Kriz W: Structure and function of podocytes: An update. *Anat Embryol* 192:385–397, 1995
28. Whiteside CI, Cameron R, Munk S, Levy J: Podocytic cytoskeletal disaggregation and basement-membraner detachment in puromycin aminonucleoside nephrosis. *Am J Pathol* 142:1641–1653, 1993
29. Schwartz MW, Bidani AK, Lewis EJ: Glomerular epithelial cell function and pathology following extreme ablation of renal mass. *Am J Pathol* 126:315–324, 1986
30. Shirato I, Sakai T, Kimura K, Tomino Y, Kriz W: Cytoskeletal changes in podocytes associated with foot process effacement in Masugi nephritis. *Am J Pathol* 148:1283–1296, 1996
31. Cohen AH, Mampaso F, Zamboni L: Glomerular podocyte degeneration in human renal disease. An ultrastructural study. *Lab Invest* 37:30–42, 1977
32. Yao X, Cheng L, Forte JG: Biochemical characterization of ezrin-actin interaction. *J Biol Chem* 271:7224–7229, 1996