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GENETIC DISORDERS – DEVELOPMENT

Expression of mitogen-activated protein kinases in human renal dysplasia

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Expression of mitogen-activated protein kinases in human renal dysplasia.

Background. We previously reported that the expression of mitogen-activated protein kinases (MAPKs) is developmentally regulated. Dysregulation of MAPKs may lead to kidney malformation. Thus, we investigated the expression of MAPKs in human renal dysplasia, one of the most common kidney malformations.

Methods. Prenatal (gestational ages 20 to 36 weeks, $N = 6$) and postnatal (2 years old, $N = 1$) dysplastic kidneys, and normal kidneys (gestational ages 19 to 34 weeks, $N = 4$) were examined. Immunohistochemical studies were performed using antibodies against extracellular signal-regulated kinase (ERK), p38 MAPK (p38), c-Jun N-terminal kinase (JNK), phospho-MAPKs (P-MAPKs), and proliferating cell nuclear antigen (PCNA). Apoptosis was detected by the TUNEL method.

Results. In dysplastic kidneys, proliferation was prominent in dysplastic tubules and also found in cyst epithelia. TUNEL staining was detected in dysplastic tubules and cysts, and occasionally in undifferentiated cells. p38 and anti-phospho-p38 (P-p38) were strongly expressed in dysplastic epithelia, but not detected in normal kidneys at any stage examined. On the other hand, JNK and P-JNK were positive in tubular epithelia of normal kidneys, whereas their expression was barely detectable in dysplastic tubules and cysts. ERK was expressed in all tubular segments, and P-ERK was detected in distal tubules and collecting ducts of normal kidneys. Dysplastic kidney epithelia stained exclusively positive for ERK and P-ERK.

Conclusions. p38 is ectopically expressed, and JNK is downregulated in dysplastic kidney epithelia. Furthermore, dysplastic epithelia are exclusively positive for ERK and P-ERK. Activated p38 and ERK may mediate hyperproliferation of dysplastic tubules resulting in cyst formation, whereas downregulated JNK expression may be the cause or the result of an undifferentiated state of dysplastic epithelia.

Renal dysplasia is one of the most common kidney malformations that lead to chronic renal failure in young children [1]. It is characterized by dysplastic tubules connected to cysts and surrounding undifferentiated tissue. Immediately around dysplastic tubules are collars of fibromuscular-like cells. Renal dysplasia is thought to be a developmental aberration of mesenchymal-epithelial transformation [2]. Thus, it is hypothesized that dysplastic tubules represent malformed collecting ducts while the surrounding tissues represent the mesenchyme that has failed to differentiate into nephrons [3, 4]. Obstruction of the urinary tract often accompanies dysplastic kidneys, and is suggested to be one of the etiologic factors. In animal experiments, prenatal obstruction of urinary tract reproduces dysplastic changes similar to human renal dysplasia [5, 6]. A disturbance of the normal pattern of ureteric bud branching also has been suggested to be the cause. The mechanism of maldevelopment of mesenchyme into nephrons, however, has not been elucidated. Increased cell proliferation in dysplastic epithelia as well as prominent apoptosis in undifferentiated mesenchyme have been reported, suggesting that deregulation of cell survival plays pivotal roles in the pathogenesis of dysplastic kidneys [7, 8].

Mitogen-activated protein kinases (MAPKs) are enzymes that play important roles in cell proliferation, differentiation, and apoptosis. We previously reported that MAPKs are developmentally regulated in rat kidney [9]. Thus, extracellular signal-regulated kinase (ERK) and p38 MAPK (p38) are strongly expressed in the developing kidney, whereas c-Jun N-terminal kinase (JNK) is detected predominantly in the adult kidney. The inhibition of ERK or p38 by specific inhibitors in organ-cultured kidneys resulted in disturbed nephron formation, ureteric bud growth, and kidney growth [10]. These results point to important roles of MAPKs in renal development. Dysregulated MAPKs, therefore, may lead to kidney malformation. In the present study, we examined the expression and localization of MAPKs and their phosophatase MAPK phosphatase-1 (MKP-1) in human renal dysplasia.

Key words: extracellular signal-regulated kinase, p38 mitogen-activated protein kinase, c-Jun N-terminal kinase, kidney malformation, development.

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Table 1. Samples

Gestational age	Sex	Renal and associated pathology
Normal kidneys *weeks*		
19	M	Spontaneous miscarriage, body stalk anomaly
21	Ambiguous	Abortion for conjoined twin
28	M	Abortion for multiple anomalies with contralateral multicystic dysplasia
34	M	Osteogenesis imperfecta
Dysplastic kidneys *weeks*		
20	Ambiguous	Spontaneous miscarriage, Potter syndrome
22	M	Abortion for bilateral multicystic dysplasia
28	M	Abortion for multiple anomalies with ipsilateral multicystic dysplasia
28	F	Abortion for bilateral multicystic dysplasia, right megaureter and ectopia
33	M	Abortion for bilateral multicystic dysplasia
36	Unknown	Abortion for bilateral multicystic dysplasia
Postnatal 2 years	F	Multiple anomaly with ipsilateral multicystic dysplasia

METHODS

Specimens

Dysplastic and control kidneys were obtained from fetuses of spontaneous miscarriages and abortions performed for severe abnormalities. Dysplastic kidneys from gestational ages 20 weeks to 36 weeks ($N = 6$) and normal kidneys from gestational ages 19 to 34 weeks ($N = 4$) were examined. One dysplastic kidney was harvested at age 2 years at autopsy. Details of these specimens are listed in Table 1. Kidney pathology was classified by gross morphology and routine histopathology. The dysplastic samples all had dysplastic tubules and undifferentiated mesenchymal tissue.

Reagents

Anti-ERK antibody (Erk1/2-CT, rabbit polyclonal IgG) was from Upstate Biotechnology (Lake Placid, NY, USA). Anti-p38 (C-20), anti-JNK (FL), and anti-MKP-1 (V-15) antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Monoclonal anti-ERK activated (P-ERK) was from Sigma (St Louis, MO, USA); anti-phospho-p38 (P-p38) and anti-phospho-JNK (P-JNK) antibodies were from New England Biolab (Beverly, MA, USA). A monoclonal antibody specific for proliferating cell nuclear antigen (PCNA)/cyclin, peroxidase-conjugated rabbit anti-mouse and peroxidase-conjugated swine anti-rabbit immunoglobulins, and DAKO® Protein K Enzyme Digestion were from DAKO A/S (Glostrup Denmark). ApopTag® Peroxydase In Situ Apoptosis Detection Kit was from Intergen Company (Purchase, NY, USA).

Immunohistochemistry

After fixation in 10% formalin, kidneys were embedded in paraffin. Immunohistochemical staining was performed on serial sections 4 μm thick, using enzyme-labeled antibody method. Paraffin sections were deparaffinized and rehydrated. Endogenous peroxide activity was quenched by incubating sections in 0.3% H_2O_2/methanol for 15 minutes. To unmask antigens, slides were boiled 100°C for 10 minutes in 10% citrate buffer (pH 6.0)/methanol. Sections were incubated with antibodies against PCNA (dilution 1:100), ERK (dilution 1:200), p38 (dilution 1:200), JNK (dilution 1:50), MKP-1 (dilution 1:100), P-ERK (dilution 1:100), P-p38 (dilution 1:100), or P-JNK (dilution 1:100) at 4°C for overnight. After incubating with secondary antibody at a concentration of 1:100, immunoreaction products were developed using 3,3′-diaminobenzidine (DAB) as the chromogen, with standardized development times. Sections were then counterstained with hematoxylin acetate or methyl green. Positive controls (brain for ERK, JNK, and MKP-1, and bone marrow for p38) were run simultaneously. Negative controls included adding saturating titers of antigen (for ERK), omitting the primary antibody or substitution of the primary antibody with rabbit serum.

Detection of apoptosis

Cells undergoing apoptosis were identified using an in situ DNA labeling method. Paraffin embedded sections were deparaffinized, and terminal deoxynucleotide transferase-mediated nick-end labeling (TUNEL) staining was performed using the ApopTag kit. Sections were counterstained with methyl green. TdT was omitted from the staining procedure in negative controls.

RESULTS

The results are summarized in Table 2. There was no difference in the expression patterns in preterm and postnatal dysplastic kidneys.

PCNA

In normal kidneys, PCNA positive cells were most abundant in the nephrogenic zone immediately under the renal capsule, which included undifferentiated mesenchymal cells, condensing mesenchyme, vesicles, comma- and S-shaped bodies, and tips of ureteric bud branches (Fig. 1A). Tubular epithelial cells in the deep cortical

Table 2. Expression of MAPKs and MKP-1 in normal and dysplastic kidneys

	p38	P-p38	JNK	P-JNK	ERK	P-ERK	MKP-1	PCNA	TUNEL
Normal kidneys									
Immature tubules/UB tips	—	—	—	—	±	±	±	+ (50%)	± (rare)
Mature tubules/CD	—	—	+	+ (distal, CD)	++	++ (distal, CD)	—	+ (rare)	—
Uninduced mesenchyme	—	—	—	—	±	±	—	+ (20%)	± (rare)
Interstitium	—	—	—	—	—	—	—	—	—
Glomeruli	—	—	± (rare)	± (rare)	+ (rare)	+ (rare)	—	+ (rare)	± (rare)
Dysplastic kidneys									
Dysplastic tubules	++	++	—	—	++	++	++	++ (40%)	+ (90%)
Cyst epithelia	+	+	—	—	+	+	+	+ (15%)	+ (80%)
Collarettes/undifferentiated cells	+	+ (rare)	—	—	+	+ (rare)	+	+ (rare)	+ (35%)

Signs denote: (—) no immunoreactivity; (±) weak immunoreactivity; (+ to ++) increasing intensity of staining present in the majority of cells in the designated population. 'Rare' indicates that <10% of cells showed positive immunostaining. Parenthesis indicates the percentage of cells positive for PCNA or TUNEL. Abbreviations are: MAPKs, mitogen-activated protein kinases; MKP-1, MAPK phosphatase-1; p-38, p-38 MAPK; P-p38, phospho-p-38 MAPK; JNK, c-jun N-terminal kinase; ERK, extracellular signal-regulated kinase; PCNA, proliferating cell nuclear antigen; TUNEL, terminal deoxynucleotide transferase-mediated nick-end labeling; UB, ureteric bud; CD, collecting ducts.

layer were occasionally stained. As previously reported, PCNA was stained intensely in dysplastic tubules and weakly in cyst epithelia of dysplastic kidneys (Fig. 1B).

TUNEL staining

In normal kidneys, TUNEL staining was faintly detectable in the nephrogenic zone (Fig. 1C). In dysplastic kidneys, apoptosis was prominent in dysplastic tubules and cysts, and occasionally found in surrounding undifferentiated cells (Fig. 1D). These findings are in accord with a previous report [7].

p38

p38 MAPK and P-p38 were not detected in control kidneys at any stage examined (Fig. 2 A, C). In striking contrast, both p38 and P-p38 were intensely stained in dysplastic epithelia (Fig. 2 B, D). p38 expression was detected also in areas of undifferentiated cells.

JNK

Contrary to p38, JNK was expressed in all the segments of mature tubules in control kidneys (Fig. 2E). P-JNK was faintly stained in distal tubules (Fig. 2G) and collecting ducts (not shown) of control kidneys. In contrast to control kidneys, JNK and P-JNK were not detected in diseased epithelia of dysplastic kidneys including the postnatal specimen (Fig. 2 F, H).

ERK

Extracellular signal-related kinase expression was weak in immature tubules and non-induced mesenchyme in the nephrogenic zone, but was detected intensely in distal tubules, collecting ducts, and to a lesser extent in proximal tubules of control kidneys (Fig. 2I). Dysplastic tubules and cysts stained positive for ERK (Fig. 2J). Surrounding undifferentiated cells also expressed ERK. P-ERK was detected in distal tubules (Fig. 2K) and collecting ducts (not shown) of control kidneys. In dysplastic kidneys, P-ERK was strongly and exclusively positive in diseased epithelia (Fig. 2L).

MKP-1

Mitogen-activated protein kinase phosphatase-1 was faintly expressed in ureteric buds of control kidneys (Fig. 2M). Dysplastic epithelia and undifferentiated cells to a lesser extent expressed MKP-1 (Fig. 2N).

DISCUSSION

The present study demonstrates that the expression of MAPKs is dysregulated in human renal dysplasia. p38 and ERK, which are thought to mediate proliferation, are up-regulated or activated, while JNK is down-regulated in dysplastic epithelia. The abnormal expression of MAPKs is associated with increased PCNA expression in dysplastic epithelia as previously reported [8]. Thus, dysregulated MAPKs may mediate hyperproliferation of dysplastic tubules leading to cyst formation.

We previously reported that p38 was expressed in the developing rat kidney [9]. The expression of p38 was most intense in the kidney at embryonic day 14 and detected up to postnatal day 1. It localized in non-induced mesenchyme, condensing mesenchyme and ureteric buds. The level and localization of phosphorylated p38 correlated with those of p38. In the normal human kidney from gestational ages 19 to 34 weeks, p38 was not detected, probably because rat and human kidneys develop over different time frames. In humans, nephrogenesis ceases at 34 weeks, whereas nephron formation continues up to postnatal day 8 in rats. In contrast to normal kidneys of comparable gestational ages, dysplastic kidneys expressed p38 and phosphorylated p38. The dysplastic kidney harvested from a two-year-old patient also showed persistent expression of p38. These results suggest that ectopically expressed p38 in dysplastic kidneys may be involved in high rate cell turnover and undifferentiated

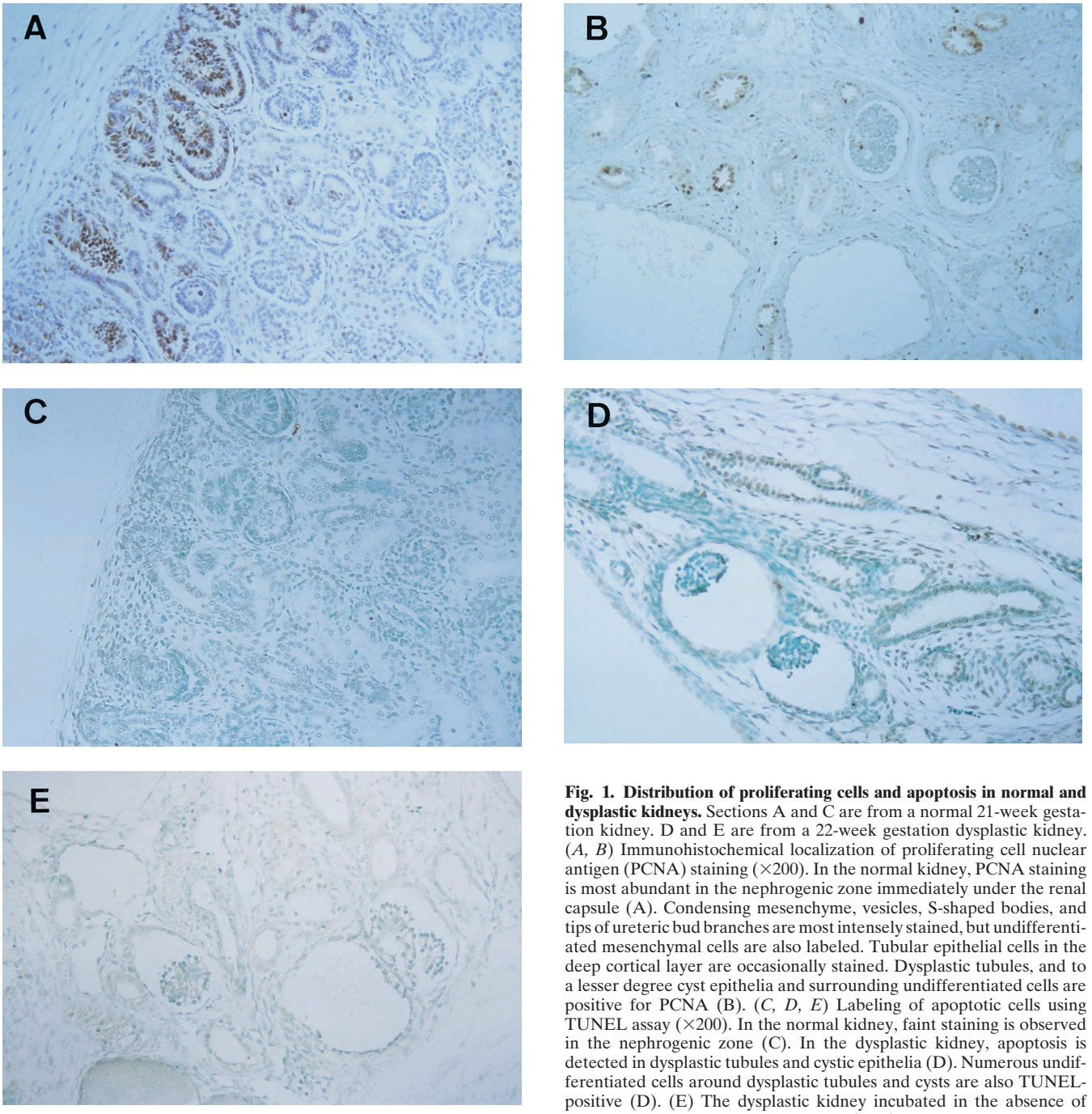

Fig. 1. Distribution of proliferating cells and apoptosis in normal and dysplastic kidneys. Sections A and C are from a normal 21-week gestation kidney. D and E are from a 22-week gestation dysplastic kidney. (*A, B*) Immunohistochemical localization of proliferating cell nuclear antigen (PCNA) staining (×200). In the normal kidney, PCNA staining is most abundant in the nephrogenic zone immediately under the renal capsule (A). Condensing mesenchyme, vesicles, S-shaped bodies, and tips of ureteric bud branches are most intensely stained, but undifferentiated mesenchymal cells are also labeled. Tubular epithelial cells in the deep cortical layer are occasionally stained. Dysplastic tubules, and to a lesser degree cyst epithelia and surrounding undifferentiated cells are positive for PCNA (B). (*C, D, E*) Labeling of apoptotic cells using TUNEL assay (×200). In the normal kidney, faint staining is observed in the nephrogenic zone (C). In the dysplastic kidney, apoptosis is detected in dysplastic tubules and cystic epithelia (D). Numerous undifferentiated cells around dysplastic tubules and cysts are also TUNEL-positive (D). (E) The dysplastic kidney incubated in the absence of terminal deoxynucleotidyl transferase (TdT).

states. Although p38 has been implicated in apoptosis, our previous study suggested that its expression correlated with proliferation rather than apoptosis in the developing kidney [9]. Furthermore, a p38 inhibitor SB203580 suppressed metanephros growth in organ culture [10]. In addition, Sodhi, Batlle and Sahai demonstrated that p38-mediated hypoxia induced cell proliferation in mesangial cells [11]. While several p38 isoforms are known to exist [12], the antibody used by us and Sodhi et al detects only p38α. Thus, p38α probably has proliferative action on renal cells.

In our previous study in the rat, ERK was expressed throughout the stages during kidney development, with the highest expression and activation levels in the fetal kidney [9]. In the normal human fetal kidney, ERK was expressed in all mature tubular segments, and phosphorylated ERK was detected in distal tubules and collecting ducts. In the dysplastic kidney, diseased epithelia were

exclusively positive for ERK and phosphorylated ERK. An explanation for the expression of phosphorylated ERK in dysplastic epithelia might be that they are of ureteric bud/collecting duct lineage. PAX2 and galectin-3, markers for this lineage, have been shown to be present in dysplastic epithelia [8, 13]. Previous studies, however, demonstrated that cysts in dysplastic kidneys were derived from Bowman's capsule, proximal tubules as well as from collecting ducts [14]. Thus, the expression of phosphorylated ERK suggests a high rate of proliferation rather than its origin, in view of the known action of ERK to promote proliferation. Winyard et al demonstrated that dysplastic epithelia maintain a high rate of proliferation along with the expression of PAX2, a potentially oncogenic transcription factor, and BCL2, an anti-apoptotic factor [8]. The authors speculated that these genes might drive the proliferation of dysplastic cysts [8]. The present study suggests that p38 and/or ERK mediate hyperproliferation of dysplastic epithelia. Whether ERK or p38 is involved in the regulation of PAX2 or BCL2, or downstream events of these genes remains to be clarified.

In addition to PAX2 and BCL2, several growth factors, their receptors, and adhesion molecules are expressed differently in dysplastic kidneys, as reviewed recently by Woolf and Winyard [2]. These growth factors include hepatocyte growth factor (HGF) and its receptor MET [15], insulin-like growth factor (IGF) [16], galectin 3 [13], and α integrin subunits [17]. We have demonstrated, to our knowledge for the first time, that signaling molecules are differentially expressed in renal dysplasia. HGF and IGF are known to activate ERK in kidney epithelial cells [18, 19]. The involvement of ERK in the signaling of adhesion molecules also has been demonstrated [20]. The role of p38 in the cellular signaling of these growth factors and adhesion molecules is less clear. Kidney malformations result from gene abnormality, obstruction of the urinary tract, maternal malnutrition and teratogenes [4]. It is conceivable that various extracellular signals affect MAPKs and lead to altered gene expression and kidney malformations. Of particular interest, ERK and p38 have been shown to be activated by cell stretch in mesangial cells [21, 22]. Urinary tract obstruction causes renal epithelial cell stretch [23]. It is possible that urinary tract obstruction may activate ERK or p38 and lead to the evolution of dysplastic kidneys.

We previously showed that JNK was expressed predominantly in the adult kidney, which suggests that JNK may be involved in the differentiation at a later stage or the maintenance of the integrity of tubular epithelia. In the present study, normal human kidneys from gestational ages 19 to 34 weeks expressed JNK, whereas dysplastic epithelia including postnatal specimen were completely negative. Epithelia lacking JNK may fail to differentiate maintaining high rate proliferation. Alternatively, the loss of JNK expression may be the result of an immature and undifferentiated state of the epithelial cells.

Although JNK as well as p38 have been implicated in apoptosis, we found no correlation between their expression and apoptosis in either normal or dysplastic kidneys. Of note, it has been suggested that the default pathway for developing renal cells is to undergo apoptosis. Only cells rescued by growth factors can survive. Apoptosis by the removal of growth factors is mediated by the phosphoinositide 3-kinase/Akt pathway [24]. Thus, apoptosis seen in the developing or dysplastic kidney may be mediated by pathways that do not involve JNK or p38.

While total and phosphorylated forms of p38 were expressed similarly, those of JNK or ERK were differentially expressed. Thus, phosphorylated JNK or ERK was detected only in distal tubules and collecting ducts. A similar pattern of differential expression of total and phosphorylated JNK or ERK was previously observed in rat adult kidneys [9]. The significance of these findings remains to be determined.

In our previous study, MKP-1 was expressed in the embryonic kidney colocalizing with p38 and ERK [9]. Undifferentiated cells around dysplastic tubules express p38, ERK, and MKP-1, displaying an expression pattern that mimicked the pattern seen in undifferentiated mesenchyme of the rat fetal kidney. These findings suggest a role of MKP-1 as a regulator of ERK and p38 in dysplastic kidneys. In normal human kidneys examined in the present study, MKP-1 was barely detectable probably due to the different time frame of development from rat kidneys.

While the mechanism of perturbed nephron formation in renal dysplasia is unknown, the up-regulated ERK or p38 may play a role. Paradoxically, our previous study demonstrated that the inhibition of p38 and ERK suppressed mesenchymal-epithelial transformation [10]. A similar scheme has been demonstrated for PAX2. Thus, too much PAX2 leads to renal dysplasia, whereas too little PAX2 results in hypoplastic kidney [25]. It is intriguing to speculate that ERK or p38 may be involved in expression of PAX2 or its downstream signaling.

A recent study by Yang et al demonstrated that transforming growth factor-β (TGF-β) is up-regulated in human renal dysplasia [26]. Since TGF-β is known to enhance epithelial-mesenchymal transformation in various tissues, the authors suggested that TGF-β might be responsible for the pathobiology of dysplastic kidneys [26]. Of interest, p38 and ERK have been shown to mediate TGF-β secretion stimulated by cell stretch or by high glucose in mesangial cells [22, 27]. It is possible that up-regulated p38 and/or ERK may mediate TGF-β up-regulation in dysplastic kidneys.

The present results suggest that MAPKs are dysregulated in human renal dysplasia. Their roles in the pathogenesis of obstructive renal dysplasia are currently under

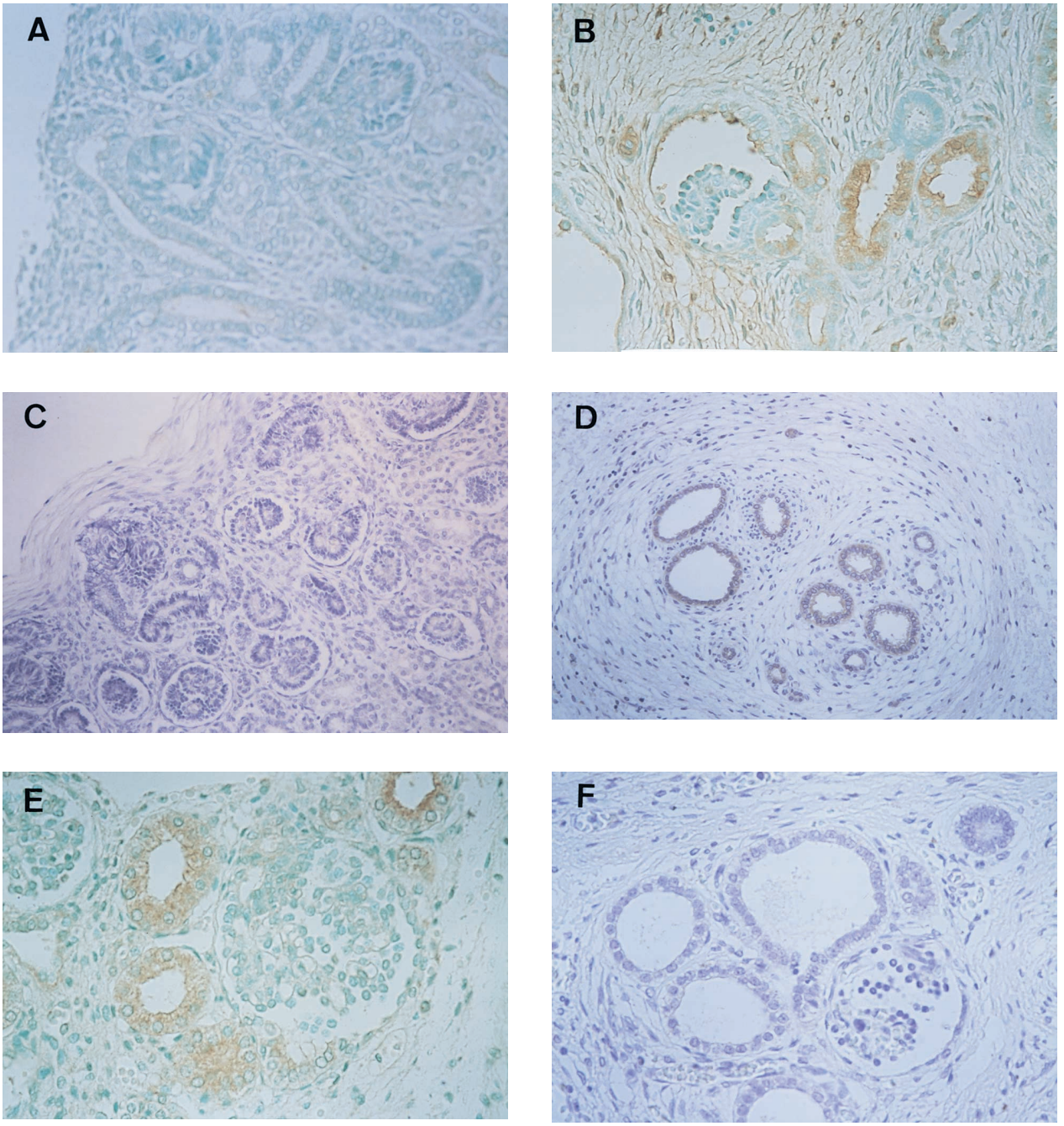

Fig. 2. Immunohistochemical localization of mitogen-activated protein kinases (MAPKs) and MAPK phosphatase-1 (MKP-1) in normal and dysplastic kidneys. Sections A, C, E, G, I, K, and M are from a normal 21-week gestation kidney. B, D, F, H, J, L, and N are from a 22-week gestation dysplastic kidney. (*A-D*) Immunohistochemical localization of p38 (×400) and P-p38 (×200). p38 is not detected in the normal kidney (A), but strongly expressed in dysplastic epithelia (B). p38-staining is also found in surrounding undifferentiated cells (B). Phospho-p38 (P-p38) is not detected in the control kidney (C), but is strongly expressed in dysplastic epithelia (D). (*E-H*) Immunohistochemical localization of JNK and P-JNK (×400). In the normal kidney, JNK is expressed in tubular epithelial cells (E). In contrast, JNK is not expressed in dysplastic tubules and cysts (F). In the normal kidney, distal tubules show weak staining for P-JNK (G). Dysplastic epithelia are entirely negative for P-JNK (H). (*I-L*) Immunohistochemical localization of ERK (×400) and P-ERK (×200). In the normal kidney, distal tubules and proximal tubules to a lesser extent stain positive for ERK (I). Immature tubules and non-induced mesenchyme are weakly stained. In the dysplastic kidney, dysplastic tubules, cystic epithelia, and occasional undifferentiated cells stain positive for ERK (J). In the normal kidney, P-ERK was detected weakly in distal tubules (K). Dysplastic epithelia stain exclusively positive for P-ERK (L). (*M, N*) Immunohistochemical localization of MKP-1 (×400). In the normal kidney, faint MKP-1 staining is detected in ureteric bud epithelia (M). In the dysplastic kidney, dysplastic epithelia show strong staining (N). Surrounding undifferentiated cells are also positive for MKP-1.

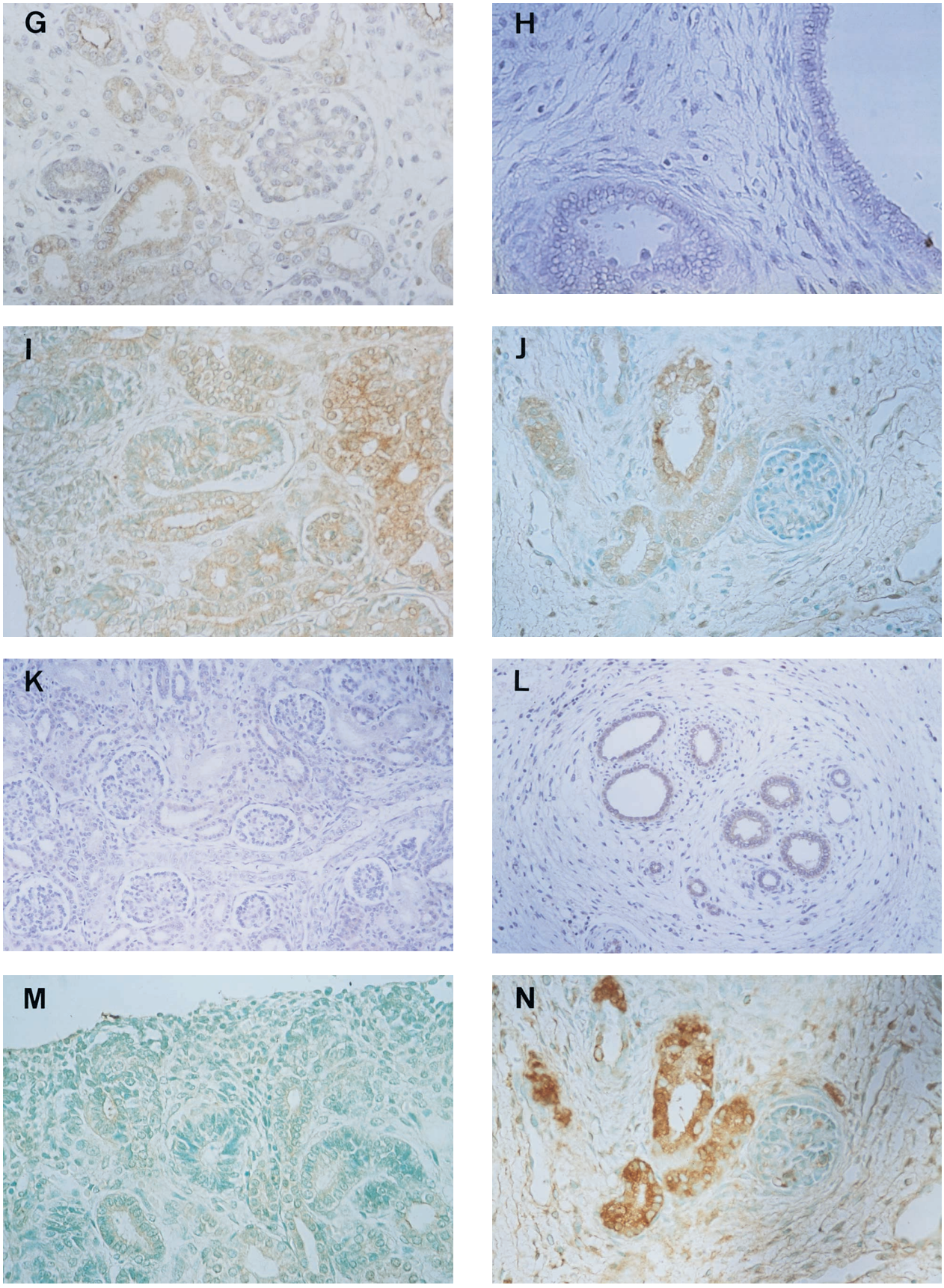

Fig. 2. (Continued)

investigation in our laboratory. Whether gene mutations, maternal nutrition, or teratogenes affect renal MAPK expression also remain to be clarified. Since pharmacological inhibition of p38 or ERK is possible, these studies may allow the development of novel therapeutic strategies for renal dysplasia.

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