Midkine Is Involved in Kidney Development and in Its Regulation by Retinoids

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Abstract. In the kidney, in which development depends on epithelial-mesenchymal interactions, it has been shown that retinoids modulate nephrogenesis in a dose-dependent manner in vivo and in vitro. Midkine (MK) is a retinoic acid responsive gene for a heparin-binding growth factor. The aim of the present study was therefore to quantify the expression of MK mRNA during renal development in the rat, to analyze the regulation of MK expression by retinoids in vivo and in vitro, and, finally, to study the role of MK in rat metanephric organ cultures. The spatiotemporal expression of MK in fetal kidney was studied. In control rats, MK expression is ubiquitous at gestational day 14, i.e., at the onset of nephrogenesis. On day 16, MK is expressed in the condensed mesenchyme and in early epithelialized mesenchymal derivatives. On gestational day 21, MK is rather localized in the nonmature glomeruli of the renal cortex. In utero exposure to vitamin A deficiency did not modify the specific spatial and temporal expression pattern of MK gene in the metanephrphs, although a decrease in mRNA expression occurred. In metanephrphs explanted from 14-d-old fetuses and cultured in a defined medium, expression of MK mRNA was found to be stimulated when retinoic acid (100 nM) was added in the culture medium. Finally, in vitro nephrogenesis was strongly inhibited in the presence of neutralizing antibodies for MK: the number of nephrons formed in vitro was reduced by ~50% without changes in ureteric bud branching morphogenesis. These results indicated that MK is implicated in the regulation of kidney development by retinoids. These results also suggested that MK plays an important role in the molecular cascade of the epithelial conversion of the metanephric blastema.

Retinoids play a critical role in fetal organogenesis by regulating the expression of genes responsible for pattern formation and morphogenesis (1). Like other embryonic processes, renal organogenesis, which is the result of interactions between the metanephric mesenchyme and the ureteric bud, depends on retinoids (2–4). In utero depletion of vitamin A or inactivation of retinoic acid receptors generates renal malformations (5–8). Using metanephric organ culture and a rat model of mild vitamin A deficiency, we clearly established that vitamin A, via its active metabolite, retinoic acid, strictly controls the number of nephrons (9,10). We then began to investigate the mechanisms of such a control, finding that the expression of the proto-oncogene c-ret was modulated in vivo and in vitro according to the retinoid environment (10,11). The proto-oncogene c-ret, a receptor tyrosine kinase present at the tips of the ureteric bud branches, is required for ureteric bud outgrowth and branching (12). Genes like Wnt11 and WTI are also controlled by retinoic acid in the metanephrphs (13). In this study, we were interested in another developmentally regulated gene, midkine (MK), a low-molecular-weight heparin-binding growth/differentiation factor (13 kD) encoded by a retinoic acid–responsive gene (14,15). MK expression increases at early stages of retinoic acid induced differentiation of embryonal carcinoma cells (16–18). In adult mice, MK mRNA is significantly expressed only in the kidney (19), but in adult rat, MK is more widely distributed (20). No data exist on the spatiotemporal pattern of MK expression in the rat embryos. In the early stages of mouse embryogenesis, MK is ubiquitously expressed. In the midgestation period, MK expression becomes restricted to tissues undergoing epithelial-mesenchymal interactions, which suggests a key role in the regulation of organ development and cell differentiation (19,21,22). The involvement of MK in epithelial-mesenchymal interactions, which characterize tooth morphogenesis, has been demonstrated (23). In vitro studies have shown that MK also promotes neurite outgrowth and survival of various embryonic nerve cells (15,24,25) and is mitogenic to certain fibroblasts, PC12 cells, and neuroectoderm cells (18,25).

In this study, we investigated the regulation of MK expression in vitamin A–deficient (VAD) fetal rat kidneys. We systematically analyzed the distribution and the expression pattern of MK in kidneys of fetuses issued from control and VAD mothers, using Northern blot and in situ hybridization techniques. We also studied the role of retinoic acid as signal mediating MK expression in rat metanephric organ culture. Finally, we addressed the question of MK implication in
nephrogenesis by analyzing the effects of neutralizing antibodies against MK protein on morphogenesis and cell differentiation in organ culture of embryonic kidney.

**Materials and Methods**

**Animals**

Female Sprague Dawley rats weighing 200 to 300 g were given free access to water and standard laboratory pellets (UAR Laboratory, Villemoyon sur Orge, France). They were caged overnight with a male, and vaginal smears were taken the following morning. The day a positive smear was obtained was designated day 0 of gestation.

Two groups of pregnant females were used, control and VAD females. Pregnant females were made vitamin A deficient by administration of an isocaloric and isoproteic diet deprived of vitamin A 6 wk before mating, as described elsewhere (10). Blood samples were taken from the cut tip of the tail, and the plasma retinol was determined by HPLC (Beckman Instruments, Inc., Fullerton, CA). The mean maternal plasma vitamin A concentration of the deficient group was 50% lower (16.8 ± 0.5 μg/dl) than in the controls (31.3 ± 0.7 μg/dl, n = 65 for both groups).

 Fetuses were removed from anesthetized pregnant females on days 14, 15, 16, 18, and 20 of gestation. Newborns and 20-d-old pups were also used. Kidneys were surgically removed from embryos, newborns, and pups, immediately frozen in liquid nitrogen, and stored at −80°C. RNA was isolated from kidneys of fetuses from one to three litters taken at 14, 15, and 16 d of gestation, from two fetuses at 18 and 20 d of gestation, and from one 20-d-old pup.

**Isolation of RNA and Northern Blot Analysis**

Total RNA was isolated from kidneys by one-step liquid-phase separation by use of the TRIzol reagent procedure (Life Technologies BRL, Grand Island, NY). RNA was subjected to agarose gel electrophoresis, and Northern blot analysis was performed as described elsewhere (26) by use of a mouse MK cDNA probe kindly provided by Prof. M. Vigny (INSERM U440, Paris, France). Signal intensity was quantified by densitometric analysis of autoradiograms (image analysis software, NIH Image) by use of hybridization with an 18S ribosomal RNA probe, to allow correction for variations in RNA loading.

**In Situ Hybridization**

*In situ* hybridization was performed in fetuses on days 14 and 16 and in the kidneys of fetuses on day 21 of gestation, as described elsewhere (26), by use of 4% paraformaldehyde-fixed, paraffin-embedded tissues and the 35S-labeled MK probe. No labeling was detected in tissue sections that were first treated with ribonuclease A and then hybridized with probe.

**Western Blot Analysis**

Recombinant human MK was obtained from medium of transfected Q2bn cells and purified by affinity column on heparin-sepharose, followed by an inverse-phase chromatography on C4. Antibodies against MK protein were generated in a chicken. Immunization was performed by two injections of 200 ng of MK, in the presence of complete, then incomplete, Freund adjuvant. Yolks were treated by 2 vol of phosphate-buffered saline and 3% of polyethylene glycol (molecular weight 6000, Merck) to precipitate phospholipids. The supernatant, filtered through cotton wool, was precipitated in presence of 12% polyethylene glycol. The precipitate was then dissolved in 1 vol of phosphate-buffered saline and dialysed extensively. Excess polyethylene glycol was removed through a Sephadex G-75 filtration column (Amersham). The protein concentration of the antibody solution was determined by absorbance at 280 nm that used IgG as a standard. Nonimmune IgY was obtained by the same method from the yolk of a nonimmunized chicken’s egg. The specificity of antibodies was checked by Western blot, first by use of recombinant human pleiotrophin (PTN), recombinant rat nerve growth factor, recombinant human MK, and a and b recombinant human fibroblast growth factor and second by use of different fetal tissue from fetuses on gestational day 15.

Western blot analysis was performed as described elsewhere (26). Protein were separated by electrophoresis in a sodium dodecyl sulfate–polyacrylamide gel (15%) to test antibody specificity regarding other growth factors and in a sodium dodecyl sulfate–polyacrylamide gel gradient (6% to 30%) to detect MK protein in fetal tissue or cultured metanephrhos. Proteins were transferred to nitrocellulose membranes (Hybond-C extra, Amersham), and the membranes were blocked by incubation for 1 h with 3% dried skim milk in Tris-buffered saline with Tween (50 mmol/L Tris-HCl, 150 mmol/L NaCl, 0.05% Tween 20 [pH 8]). MK protein was detected by incubating with antibody 1/5000 diluted in 3% dried skim milk in Tris-buffered saline with Tween for 1 h at room temperature. A peroxidase-coupled anti-IgY (Jackson ImmunoResearch laboratories, West Grove, PA) was used as the secondary antibody. Antigen-antibody complexes were detected by enhanced chemiluminescence as recommended by the manufacturer (Amersham). Each membrane was labeled with a monoclonal anti–β-actin antibody (Sigma) to normalize the amount of protein loaded, as described elsewhere (26). Bands were quantified by densitometry that used image analysis software (NIH image).

**Metanephric Organ Culture**

Metanephrhos were cultured on a defined serum-free medium as described elsewhere (9). A stock solution of retinoic acid (10 mM, Sigma) was prepared in ethanol and stored in the dark at −20°C, and dilutions were made daily in culture medium. The antibody used in culture is a chicken anti-mouse MK polyclonal antibody 1/100 diluted in culture medium.

One metanephros from each fetus was grown in control medium, and the opposite metanephros was grown in the same medium with either retinoic acid (100 nM) or MK antibody (100 μg/ml). IgY nonimmune purified fraction was added in control medium for experiments with neutralizing antibodies to MK. All media were changed daily.

**Glomerular and Tubular Structure Assessment and Protein Content in Metanephric Organ Culture**

Nephrons formed *in vitro* were counted after labeling the glomerular structures in the explanted metanephor by use of specific lectin-binding sites located on podocyte membranes (27). Briefly, the explanted metanephor were fixed in 2% paraformaldehyde phosphate-buffered saline, detached from the filter, permeabilized with saponin, and labeled with rhodamine-coupled peanut agglutinin, which stains glomeruli. The use of another lectin, Dolichos biflorus agglutinin, fluorescein coupled, allowed us to analyze the ureteric bud arborization and to count the end buds. The growth of the explanted metanephor was then determined by measurement of their protein content. The labeled metanephor were placed in individual tubes that con-
tained 0.5 ml distilled water, rinsed, and sonicated for 15 s. The protein content was measured according to the procedure of Lowry et al. (28) as modified by Larson et al. (29), with the use of bovine serum albumin as a standard.

**Statistical Analyses**

All values are expressed as means ± SEM. Differences between developmental stages were calculated by ANOVA. Control and experimental groups, at the same stage, were compared by Student’s unpaired t test. Student’s paired t test was used to compare in vitro data. The threshold of significance was taken to be \( P < 0.05 \).

**Results**

**Specificity of the Purified Anti-MK Antibody**

The purified chicken anti-MK antibody reacted strongly with MK, and a unique band of 15 kD was detected on Western blots (Figure 1A, lane 4); however, it did not react with recombinant human PTN (lane 1), even though MK and PTN have \( \sim 50\% \) sequence identity (30,31). Anti-MK also did not react with nerve growth factor (lane 2) and a fibroblast growth factor (lane 4) or b fibroblast growth factor (lane 5), which are other typical heparin-binding growth factors. We conclude that the anti-MK antibody has high specificity. Figure 1B reported MK expression in a broad range of molecular-weight proteins from different fetal tissue homogenates from fetuses on gestational day 15. An unique band of 15 kD was also detected.

**Vitamin A Controls MK Expression in Fetal Kidney**

MK expression was ubiquitous in 14-d-old control fetuses. In kidney, it was equally displayed between the mesenchymal and epithelial tissue (Figure 2A). From day 16 onward, MK expression becomes restricted to brain (neopallial cortex), stomach, intestine, lung, and kidney. In the latter, on day 16 of

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**Figure 1.** Specificity of the purified anti-midkine (MK) antibody by Western blotting. (A) Cross-reaction with other growth factors in 15% sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE). Lane 1, Recombinant human pleiotrophin (PTN); lane 2, recombinant rat nerve growth factor (NGF); lane 3, recombinant human MK; lane 4, recombinant human a fibroblast growth factor (FGF); and lane 5, recombinant human bFGF. Of each protein factor, 0.5 \( \mu \)g was loaded. (B) MK detection in fetal tissues on gestational day 15 in a 6% to 30% SDS-PAGE gradient. Lane 1, kidney; lane 2, liver; lane 3, lung; lane 4, testis; and lane 5, spinal cord. Of each tissue homogenate, 30 \( \mu \)g of protein was loaded. The positions of protein markers are shown on the left (as kD).

**Figure 2.** Expression of MK mRNA in the kidney during embryonic development as detected by in situ hybridization. Sections of kidneys from (A) 14-, (B) 16-, and (C) 21-d-old fetuses were hybridized with an \(^{35}\)S-labeled MK probe and counterstained with hematoxylin-eosin. (A) Section of metanephros showing a labeling of both ureteric bud (\( \rightarrow \)) and mesenchyme (\( \nearrow \)). (B) Section of kidney showing a strong MK expression in the nephrogenic zone, in the mesenchyme (\( \swarrow \)) and in the nonmature glomeruli (\( \leftarrow \)). In contrast, low staining was observed in the mature tubule in the profound zone of the kidney (\( \leftrightarrow \)). (C) Section of kidney disclosing a decreased expression of MK in the mesenchyme (\( \swarrow \)). The expression was restricted to the nonmature glomeruli (\( \leftarrow \)). In the mature glomeruli, the MK expression was weak (\( \leftrightarrow \)). Magnification, \( \times 400 \).
gestation, MK was mainly found in the metanephrogenic zone, i.e., in the condensed mesenchyme, in the ureteric bud branches, and in nephron anlagen. The interstitium was not labeled, and the expression in the collecting ducts in the profound zone of the kidney was very low (Figure 2B). On day 21, MK was still strongly expressed in the immature glomeruli but was decreased in the mesenchyme. Its expression was also weak in the mature glomeruli (Figure 2C). MK mRNA was detected as a unique 0.9-kb band by Northern blot hybridization analysis. Densitometric analysis was made taking control fetuses of 14 d as 100%. MK expression was maximal on the early stages of nephrogenesis and then declined to reach a very low level after birth. The decrease was 65% on fetal day 16 and reached 90% on postnatal day 20 (Figure 3). In VAD fetuses, a similar level of MK expression was observed at gestational day 14. From day 16, a decrease in MK mRNA occurred that is of ~50% for the whole period studied. Exposure to vitamin A deficiency in utero did not modify the specific spatial and temporal expression patterns of MK. However, consistent with Northern blot results, the MK expression decreases from day 16 in the metanephrogenic zone and in the S-shaped bodies of VAD fetal kidneys, compared with that in controls (Figure 4, A through D). On day 21, MK expression was weak in the mature glomeruli of both control and VAD fetuses (Figure 4, E and F).

Retinoic Acid Stimulates MK Expression in Metanephric Organ Culture

We quantified the changes in MK expression induced by retinoic acid in the metanephros explanted from 14-d-old fetuses. Retinoic acid was added to the culture medium at the final concentration of 100 nM, which increased by ~40% the number of end buds after 2 d of culture and led to a threefold increase in the number of glomeruli after 6 d of culture. Densitometric analysis of Northern blots shows that, under these conditions, MK mRNA expression was increased by ~70% and 100% after 24 and 48 h of culture, respectively (Figure 5A). The amount of protein increased significantly, by ~30%, after 2 d of culture (Figure 5B).

Neutralizing Antibodies to MK Impaired In Vitro Nephrogenesis

The addition of neutralizing antibodies to MK alters nephrogenesis in E14 metanephros compared with the opposite metanephros cultured in the presence of the IgY nonimmune-purified fraction as control conditions (Figure 6). The number
of glomeruli counted after labeling (Figure 6, A and B) was not changed by the presence of the antibody (Figure 7A), whereas ureteric bud branching morphogenesis assessed on explanted metanephroi grown for 2 d and subsequently labeled with Dolichos biflorus agglutinin was not changed by the presence of the antibody (Figure 6, C and D). This was confirmed by the lack of difference in the number of end buds (Figure 7B). No effect of MK antibody was observed on the surface area (Figure 6) or total protein content per metanephros (Figure 7C) in E14 metanephric kidneys cultured for 4 d.

Discussion

The effect of retinoids on the expression of several genes known to play a key role in fetal organogenesis has been demonstrated elsewhere. Particularly in VAD animals, genes involved in the retinoic acid signaling pathway and other genes encoding for transcription factors, growth factors, and extracellular matrix components have been reported to be regulated by vitamin A status (32–37). As already mentioned, the protooncogene c-ret is down-regulated in the metanephros of the VAD fetus (10). In this study, both Northern blot and in situ hybridization analysis of kidneys of VAD fetuses clearly show that vitamin A status also regulates MK expression during nephrogenesis. In addition, a significant increase in MK mRNA expression occurred within 24 h of retinoic acid stimulation in metanephric organ culture, which suggests that MK regulation is directly mediated by retinoic acid. This hypothesis is supported by the mapping and characterization of a retinoic acid–responsive enhancer ~900 nt upstream of the MK mRNA transcription start site reported for the mouse gene (38). The core element was mapped to positions −976 to −951. The binding of a retinoic acid receptor heterodimer DR5 type RARE was verified by use of gel-shift analysis. Transfection of a MK promoter/CAT reporter construct in EC, F9, and HM-1 cells showed a fivefold to tenfold induction of CAT activity by retinoic acid at the same concentration as that used in this study (100 nM).

Spatiotemporal localization of MK mRNA has been reported in the whole mouse embryo, but only partial data exist about the developing kidney (19,22). No data exist in the rat embryo. The present data, which show that the in vivo pattern of MK expression is developmentally regulated in the metanephros, support the hypothesis of a role of MK in nephrogenesis. The ubiquitous expression of MK determined on day 14 of gestation, i.e., at the onset of kidney organogenesis, becomes restricted to the metanephrogenic mesenchyme as nephrogenesis proceeds. At the end of fetal nephrogenesis, expression reached very low level. This suggests that MK may play a role as a survival factor for mesenchymal cell compartment, as proposed by Burrow and colleagues (39). We also found MK transcript accumulation in early epithelialized mesenchymal derivatives, which suggests that MK promotes the cell proliferation and/or differentiation during the first stage of the epithelial conversion of the metanephric blastema. The role of MK in epithelialization of the mesenchyme also emerged from anti-MK antibody in vitro experiments that have shown an important alteration of nephrogenesis leading to a reduction in the number of nephrons. However, no modification of the growth of the explant and of the ureteric bud branching morphogenesis has been observed. Misiadis et al. (40), using
polyclonal rabbit antibody against MK, were unable to show any role for MK in nephrogenesis. This difference could be attributed to the different antibody affinities for MK or/and to species differences. It is worthwhile to note that addition of MK to embryonic mouse lung explants stimulated mesenchymal tissue with no effect on branching morphogenesis (41). Mice lacking the MK gene present no abnormalities in the organs, including lung and kidney. In fact, genetic redundancy due to genes of the same family can severely limit the power of this gene knockout study (42). Indeed, PTN, another heparin-binding growth factor, is found in epithelio-mesenchymal organs such as kidney and lung (21). Finally, the fact that the presence of anti-MK antibodies in the culture media did not change the ureteric bud branching morphogenesis, together with the fact that MK is regulated by retinoic acid, suggest that this latter may have an effect on the epithelial conversion of the metanephric blastema. Until now, only an effect of retinoic acid on the ureteric bud branching capacity had been reported (9).

Other factors that regulate MK expression have been reported elsewhere (43,44). Among them, the Wilms’s tumor suppressor gene WT1 plays a key role in nephrogenesis (45). During normal kidney development, WT1 is maximally expressed in the mature glomeruli (46–48). In this study, we have shown that the MK gene is expressed in condensing metanephric mesenchyme and in early epithelial structures derived from the mesenchyme; then, it is down-regulated as the nephron matures. It was not detected in mature glomeruli, as described elsewhere, in the adult kidney (49). The down-regulation of MK is coincident with the up-regulation of WT1 in the developing nephron, which suggests that MK could be a target gene for WT1. Using Wilms’s tumor cells, Adachi et al. (43) provided evidence that a WT1 gene product indeed suppresses MK gene expression through binding to its promoter region. Similarly, WT1 suppresses pax2 expression during late stages of nephron differentiation (50).

Taken together, the in vivo and in vitro data provide evidence for the implication of MK in molecular process of nephrogenesis. They indicate that MK is a target gene for retinoids and is involved in the stimulating effect of retinoids on nephrogenesis.
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References


Figure 7. Quantitative analysis of in vitro development of kidneys from 14-d-old embryos cultured in absence (open bars) or in the presence (hatched bars) of neutralizing antibody to MK (MK Ab). (A) Differentiation was analyzed by counting the total number of glomeruli present within the metanephroi. (B) Branching morphogenesis of the ureteric bud was analyzed by counting the number of end buds. (C) Growth was assayed by protein content determination. **P < 0.01 compared with controls.


