Role of membrane-type matrix metalloproteinase 1 (MT-1-MMP), MMP-2, and its inhibitor in nephrogenesis

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Kanwar, Yashpal S., Kosuke Ota, Qiwei Yang, Jun Wada, Naoki Kashihara, Yufeng Tian, and Elisabeth I. Wallner. Role of membrane-type matrix metalloproteinase 1 (MT-1-MMP), MMP-2, and its inhibitor in nephrogenesis. Am. J. Physiol. 277 (Renal Physiol. 46): F934–F947, 1999.—Extracellular matrix (ECM) proteins, their integrin receptors, and matrix metalloproteinases (MMPs), the ECM-degrading enzymes, are believed to be involved in various biological processes, including embryogenesis. In the present study, we investigated the role of membrane type MMP, MT-1-MMP, an activator pro-MMP-2, in metanephric development. Also, its relationship with MMP-2 and its inhibitor, TIMP-2, was studied. Since mRNAs of MT-1-MMP and MMP-2 are respectively expressed in the ureteric bud epithelia and mesenchyme, they are ideally suited for juxtacrine/paracrine interactions during renal development. Northern blot analyses revealed a single ∼4.5-kb mRNA transcript of MT-1-MMP, and its expression was developmentally regulated. Inclusion of MT-1-MMP antisense oligodeoxynucleotide (ODN) in the culture media induced dysmorphogenic changes in the embryonic metanephros. MMP-2 antisense ODN also induced similar changes, but they were relatively less; on the other hand TIMP-2 antisense ODN induced a mild increase in the size of explants. Concomitant exposure of MT-1-MMP and MMP-2 antisense ODNs induced profound alterations in the metanephro. Treatment of TIMP-2 antisense ODN to metanephré exposed to MT-1-MMP/MMP-2 antisense notably restored the morphology of the explants. Specificity of the MT-1-MMP antisense ODN was reflected in the selective decrease in its mRNA and protein expression. The MT-1-MMP antisense ODN also resulted in a failure in the activation of pro-MMP-2 to MMP-2. These findings suggest that the trimacromolecular complex of MT-1-MMP:MMP-2:TIMP-2 modulates the organogenesis of the metanephros, conceivably by mediating paracrine/juxtacrine epithelial:mesenchymal interactions.

membrane-type matrix metalloproteinase-1; matrix metalloproteinase-2; embryonic development

DIFFERENTIATION, MIGRATION, and proliferation of pluripotent cells are the series of interlinked processes that heavily influence the organogenesis during embryonic life. These processes are modulated by a diverse group of macromolecules, including extracellular matrix (ECM) glycoproteins, their receptors, i.e., integrins, and ECM-degrading enzymes and their inhibitors (16, 33, 35, 45, 48, 81). These ECM macromolecules are known to exert a considerable influence on the epithelial:mesenchymal interactions that are prevalent during embryogenesis, and thus they are believed to regulate the morphogenesis of various organs (4, 15, 17, 19, 26, 61, 66, 82). Since remodeling of the ECM is influenced by the ECM-degrading enzymes, e.g., matrix metalloproteinases (MMPs), the latter could be regarded as the integral component of the process of morphogenesis/organogenesis (44, 84). The organs that are prototypic of the epithelial:mesenchymal interactions include mammary and salivary glands, lung, and metanephros.

In the latter, nephrogenesis ensues following the reciprocal interaction of epithelial ureteric bud branches with the loose metanephric mesenchyme, followed by inductive conversion of the mesenchyme into an epithelial phenotype with subsequent formation of nascent nephrons (30, 66). Such cell:matrix interactions, modulated in part by the ECM-degrading enzymes, are conceivably essential to sustain organogenesis of the embryonic mammalian metanephros.

Some of the ECM-degrading enzymes are zinc metal-dependent proteolytic enzymes, the latter are commonly known as MMPs. The MMPs have been implicated in remodeling of ECM during various physiological processes and in pathological states (5, 8, 42, 44, 71). At present, there are about 16 known human MMPs that can be classified into distinct groups: interstitial collagenases, stromelysins, gelatinases, elastases, RXKR secreted type MMPs, and RXKR membrane type MMPs (16, 41, 43, 52, 56, 64, 71, 74, 75, 86). MMPs of the last two groups share the RXKR (arginine-arginine/glutamic acid-lysine-arginine) recognition motif for the Golgi-associated proteinase, furin (53), that appears to regulate proenzyme processing to active enzyme for these two groups (54, 56). MMPs have common structural characteristics, which include pre/propeptide, hinge, hemopexin-like domains (except MMP-7), and catalytic domains; the latter domain is the zinc binding site (6). The gelatinases (MMP-2 and -9) have additional fibronectin type-II-like domains, whereas the membrane-type MMPs (MT1-4-MMPs) have linker transmembrane and cytoplasmic regions (43).

The gelatinases, MMP-2 and MMP-9, are distinctive members of the MMP family in that they form proenzyme complexes with the endogenous tissue inhibitors of metalloproteinases (TIMPs). Pro-MMP-2 selectively complexes with TIMP-2, and pro-MMP-9, in monomeric form, complexes with TIMP-1 (27, 69, 72). The role of these proenzyme-inhibitor complexes in regulation of ECM turnover is not clear; however, their formation may account, at least in part, for selective inhibition of MMP-2 by TIMP-2. A similar selective inhibition by TIMP-2 has also been reported for MT-1-MMP (65). The activation of many of the soluble MMPs can be initiated by plasmin (16, 46, 50). MMP activation is accompanied...
by a decrease in the molecular mass of about 8 kDa after removal of the profragment. However, soluble pro-MMP-2 is unique in that this enzyme is not activated by plasmin in solution but requires a specific cell surface mechanism for processing and activation of the proenzyme and proenzyme-TIMP-2 complexes (3, 9, 10, 83). These studies subsequently led to the identification of MT-1-MMP and demonstration that this membrane-type matrix metalloproteinase activates pro-MMP-2 and pro-MMP-2/TIMP-2 complexes (32, 64). Conceivably, the interaction of these three macromolecules, MT-1-MMP:MMP-2:TIMP-2, may ultimately influence various biological processes related to ECM remodeling (49).

Since pro-MMP-2 mRNA is expressed in the mesenchymal cells (59, 76) and MT-1-MMP mRNA is mainly in the epithelial cells (52, 76), it is therefore conceivable that paracrine/juxtacrine interactions may be needed to facilitate MT-1-MMP-activation of pro-MMP-2 and initiation of ECM remodeling (79). Such a scenario would be ideally suited for epithelial:mesenchymal interactions that are prevalent during embryonic development. In view of these considerations, studies were initiated to assess the conjoint role of these two metalloproteinases and their selective inhibitor TIMP-2 in the organogenesis of the embryonic metanephros.

**MATERIALS AND METHODS**

Animals. Paired male:female mating of ICR mice (Harlan Sprague Dawley, Indianapolis, IN) was carried out, and the kidneys from fetuses at days 13, 17, and 19 (newborn) of gestation were harvested. In addition, kidneys from 1-, 2-, and 3-wk-old mice were obtained.

Northern blot analyses. Total RNA from mice kidneys was isolated by the guanidinium isothiocyanate-CsCl centrifugation method (13), and poly(A)⁺ RNA was selected by oligo(dT) cellulose chromatography. Five micrograms of mRNA was glyoxalated and subjected to 1% agarose gel electrophoresis in 10 mM sodium phosphate buffer, pH 7.0. The electrophoresed mRNA was transferred to a nylon membrane filter (Amersham). The nylon membrane mRNA blot was then hybridized with [α-³²P]dCTP-labeled mouse MT-1-MMP cDNA (52) and β-actin cDNA (GenBank accession no. M62174; ATCC, Rockville, MD). The filter blot was washed at high-stringency conditions with 0.1× SSC and 0.1% SDS at 60°C, and an autoradiogram was prepared.

Antisense experiments. The antisense experiments were performed to determine the role of MT-1-MMP in organogenesis of embryonic kidneys in conjunction with that of MMP-2 and TIMP-2. First, nonsense-, sense-, and antisense-phosphorothioated oligodeoxynucleotides (ODNs) were synthesized by automated solid-phase synthesizer (Biotech Facility, Northwestern University), and purified by high-performance liquid chromatography. A 32-mer antisense ODN (5'-CGCCTGGCT-GAGGATGC-3') was selected from within the open reading frame of the cloned MT-1-MMP (52). Similarly, antisense ODNs for mouse MMP-2 (25-mer) TIMP-2 (32-mer) were synthesized. Their nucleotide sequences were derived from within the open reading frame, and they were as follows: 5'-CAGCACAAAAGAGTGGCAACTCC-3' for MMP-2 (59) and 5'-CTGCAATGGAAAAGCTTGGTGGTGCC-3' for TIMP-2 (67). Their specificity for the target nucleotide sequences was established by S1 nuclelease protection assays as described previously (28, 80; see results). Two nonsense 31-mer phosphorothioated ODNs were also synthesized, and their sequences were as follows: 5'-TAATGATAGAATGTGATGATAAGATT-3' and 5'-GATCAGCGATCGATCGATCGATCGATCGAT-3'. The ODNs (antisense and nonsense) did not exhibit any significant homology with other mammalian nucleotide sequences available in the GenBank database.

About 1,200 mouse embryonic kidneys at day 13 of gestation were harvested and maintained in an organ culture system for 4 days, as detailed previously (37, 80). Briefly, the harvested metanephric explants were placed on an 0.8-µm pore size filter, floated onto a serum-free medium composed of equal volumes of DME and Ham's nutrient mixture F-12, with penicillin (100 µg/ml), streptomycin (100 µg/ml), and transferrin (50 µg/ml) (Sigma Chemical, St. Louis, MO). The ODNs were added to the culture media daily at a concentration of 0.5 µM. At this concentration, the ODNs retain their specificity with no discernible cytotoxic effects (12, 28, 80). The metanephric explants (~200 kidneys per variable, i.e., control, antisense, and sense or nonsense) were processed for light microscopy, quantitative RT-PCR analyses, and immunofluorescence, immunoprecipitation, and zymographic studies. For light microscopy, the sections from the midplane of the embryonic kidneys with a maximum number of ureteric bud iterations, including both the poles and the hilum, were evaluated (37, 80). To investigate the regulation of MMP-2 activation and MMP-2 activity, as well as the related roles of MT-1-MMP and TIMP-2 in metanephric development, the explants were concomitantly exposed to either MT-1-MMP and MMP-2, MT-1-MMP and TIMP-2, or MMP-2 and TIMP-2 antisense ODNs. The treated explants were processed for light microscopy as described above.

Quantitative RT-PCR analyses of mRNA of antisense-treated metanephroi. To assess the effect of antisense ODNs on the mRNA expression, competitive RT-PCR was performed. Total RNAs were isolated from ~50 explants, per variable, by acid guanidinium isothiocyanate-phenol-chloroform extraction method (14). Extracted RNAs were digested with 100 U of RNase-free DNase (Boehringer Mannheim, Indianapolis, IN) containing 1 U of RNase inhibitor (Promega). After another phenol-chloroform extraction, the RNAs were ethanol precipitated. About 25 µg of total RNAs, from each variable, was first-strand cDNA synthesis using MMLV-RT and oligo(dT) as a primer. The cDNAs from different variables were suspended in 25 µl of autoclaved water and kept at ~70°C.

For the analyses of MT-1-MMP, the respective sense (MT-SE) and antisense (MT-AS) primers were as follows: 5'-CTGATGACGATCGCCGTGGCATCC-3' and 5'-GGCGTCT- GAAGAAGAACGACGCGAGG-3' (52). For β-actin, the respective sense (β-SE) and antisense (β-AS) primers were 5'-GAGCACCATGGAAGATCTCGG-3' and 5'-GAGGATGC- GGCAAGTGCG-3' (78). With these primers, the PCR products of an expected size of 879 bp for mouse MT-1-MMP and 461 bp for β-actin were generated. The 879-bp PCR product was used for the preparation of a "competitive DNA template" for MT-1-MMP. It was first ligated into pCR II (Invitrogen) and sequenced for determining its orientation. The metanephric explants (50 explants) were then placed on an 0.8-µm pore size filter, floated onto a serum-free medium composed of equal volumes of DME and Ham's nutrient mixture F-12, with penicillin (100 µg/ml), streptomycin (100 µg/ml), and transferrin (50 µg/ml) (Sigma Chemical, St. Louis, MO). The ODNs were added to the culture media daily at a concentration of 0.5 µM. At this concentration, the ODNs retain their specificity with no discernible cytotoxic effects (12, 28, 80). The metanephric explants (~200 kidneys per variable, i.e., control, antisense, and sense or nonsense) were processed for light microscopy, quantitative RT-PCR analyses, and immunofluorescence, immunoprecipitation, and zymographic studies. For light microscopy, the sections from the midplane of the embryonic kidneys with a maximum number of ureteric bud iterations, including both the poles and the hilum, were evaluated (37, 80). To investigate the regulation of MMP-2 activation and MMP-2 activity, as well as the related roles of MT-1-MMP and TIMP-2 in metanephric development, the explants were concomitantly exposed to either MT-1-MMP and MMP-2, MT-1-MMP and TIMP-2, or MMP-2 and TIMP-2 antisense ODNs. The treated explants were processed for light microscopy as described above.

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After confirming the nucleotide sequence (63), the clones with sense orientation were selected, linearized with appropriate restriction enzyme digestion, and used as a competitive DNA template for MT-1-MMP mRNA analyses by RT-PCR. The construction of a competitive 224-bp DNA template for \( \beta \)-actin has been described previously (29, 80; GenBank accession no. U17140).

The mRNA analyses of MT-1-MMP (competitive truncated DNA template) were carried out by RT-PCR, and their competitive DNA templates were prepared in a manner similar to that for MT-1-MMP. First, respective 576-bp and 568-bp PCR products of MMP-2 and TIMP-2 were prepared in a manner similar to that for MT-1-MMP. The respective sense and antisense primers for MMP-2 were 5'-CCCTATCTACCTCTCACCCAAGAAC-3' (M2-SE) and 5'-CATTCCAGAGGTCTGTCCGATGAGC-3' (M2-AS), with an expected size of the PCR product of 576 bp (59). The respective sense and antisense primers for TIMP-2 were 5'-GCAAAGGCGGTTTTGCAATG-CAGACG-3' (T2-SE) and 5'-GCTTCTTGCTGGGCTCCTCGATGTCGAAG-3' (T2-AS), with an expected size of the PCR product of 568 bp (67).

To generate their “competitive DNA templates”, the PCR products were digested with restriction enzymes, i.e., SacI for MMP-2 and KpnI for TIMP-2, followed by ligation of the truncated products in PCR II plasmid. The respective sizes of truncated MMP-2 and TIMP-2 DNA templates were 354 bp and 400 bp. After cloning and confirming their nucleotide sequences (63), the plasmids were linearized and used as competitive DNA templates for MMP-2 and TIMP-2 mRNA analyses.

For quantitative RT-PCR analyses, a fixed amount of cDNAs (1 µl) from antisense- and nonsense-ODN-treated metanephroi and serial logarithmic dilutions of the competitive template DNA (500 ng/µl) of MT-1-MMP were coamplified (22). The reaction mixture included 5 µl of 10× PCR buffer (100 mM Tris·HCl, pH 8.3, 15 mM MgCl₂, 500 mM KCl, and 0.01% gelatin), 250 µM of each dNTP, 1 µM of sense and antisense primers, and 1 U Taq polymerase (Perkin-Elmer, Norwalk, CT) in a total volume of 50 µl. The amplification reaction was carried out for 30 cycles in a DNA Thermal Cycler (Perkin-Elmer); each cycle consisting of denaturation at 94°C for 1 min, annealing at 60°C for 1 min, and extension at 72°C for 1 min. The PCR products of wild-type and mutant MT-1-MMP (competitive truncated DNA template) were analyzed by 2% agarose gel electrophoresis and photographed using an instant positive/negative film (Kodak, Rochester, NY). The autoradiograms were prepared.

For immunoprecipitation, 100 explants, per variable, were radiolabeled with \( [35S] \) methionine (0.25 mCi/ml) for 12 h prior to the termination of the culture. After a brief rinse with culture media, they were lysed in an extraction buffer (50 mM Tris·HCl, pH 7.5, 50 mM NaCl, 10 mM EDTA, 0.2% Na₂N₃, 1% Triton X-100, 0.25 mM dithiothreitol, 10 mM benzamidine-HCl, 10 mM \( \epsilon \)-aminocaproic acid, and 2 mM phenylmethylsulfonyl fluoride) by shaking vigorously for 2 h at 4°C. The extract was centrifuged at 10,000 g for 30 min at 4°C, and the supernatant was saved. Incorporated radioactivities in the supernatants were determined by trichloroacetic acid precipitation. Equal amounts of incorporated radioactivity (5 × 10⁶ dpm), in the antisense, nonsense, and control groups, were used for immunoprecipitation. Immunoprecipitation was performed by adding 5 µl of polyclonal anti-MT-1-MMP antibody to 0.5 ml (5 × 10⁶ dpm) of the supernatant. The mixture was gently stirred in an orbital shaker for 15 h at 4°C. After addition of 80 µl of protein-A Sepharose 4B (Pharmacia LKB Biotechnology, Piscataway, NJ), the antibody-antigen mixture was further incubated for 1 h at 4°C. The complex was then microcentrifuged for 10 s, and washed three times with the extraction buffer. The immunoprecipitated complex was dissolved in a sample buffer (4% SDS, 150 mM Tris·HCl, pH 6.8, 20% glycerol, 0.1% bromophenol blue, and 10% \( \beta \)-mercaptoethanol), boiled for 5 min, and subjected to 10% SDS-PAGE. The gels were fixed in 10% acetic acid and 10% methanol, treated with 1 M salicylic acid, and vacuum dried, and autoradiograms were prepared.

MMP-2 gelatin zymography in antisense-treated metanephroi. To determine the gelatinolytic activity of MMP-2 in antisense-treated metanephroi, zymography was performed (25, 34, 58). Ten microliters of metanephric organ culture medium was mixed with an equal volume of sample buffer (100 mM Tris·HCl, pH 6.8, 20% glycerol, 0.2% bromophenol blue) and incubated for 20 min at 37°C to dissociate MMP-TIMP complexes (11). Samples were then subjected to electrophoresis in a gel containing 10% polyacrylamide, 0.1% SDS, and 1 mg/ml of gelatin at 4°C. The gels were then washed in a solution containing 2.5% Triton X-100, 100 mM Tris·HCl, pH 7.6, 5 mM CaCl₂, and 1 µM ZnCl₂ for 1 h at 24°C to activate proenzyme by removing SDS. This was followed by an incubation in a reaction buffer consisting of 100 mM Tris·HCl, pH 7.6, 5 mM CaCl₂, and 1 µM ZnCl₂ for 18 h at 37°C. The gels were fixed in 10% acetic acid and methanol and stained with 0.1% Coomassie brilliant blue R-250 (Sigma Chemical). The MMP-2 activity was detected by the appearance of clear bands in the stained gel.

RESULTS

Developmental mRNA expression of mouse MT-1-MMP. Northern blot analyses of mRNA, isolated from kidneys during the embryonic and neonatal periods, revealed a single 4.5-kb transcript (Fig. 1A, top). The mRNA expression in the kidney steadily increased from day 13 to 19 of gestation, and it gradually decreased during the postnatal period, suggesting that MT-1-MMP is developmentally regulated during the organogenesis of the metanephros. The highest mRNA expres-
investigate the role of MT-1-MMP in renal organogenesis.

Role of MT-1-MMP in embryonic renal development (antisense experiments). To assess the role of MT-1-MMP in embryonic organ development, antisense experiments were performed. In addition, a limited number of experiments were carried out with MMP-2 and TIMP-2 antisense ODNs as well, to establish their biological relevance with MT-1-MMP in the context of renal development. The specificity of the antisense ODNs was established by the S1 nuclease protection assays. A single band of radioactivity, corresponding to their respective sizes, i.e., MT-1-MMP = 32-mer, MMP-2 = 25-mer, and TIMP-2 = 32-mer, was observed at 35°C and 40°C hybridization temperatures (Fig. 1B). No band of radioactivity was seen in the control (nonsense ODN) samples at either hybridization temperatures.

Effect of antisense ODN on the morphology of renal explants. The alterations induced by the antisense ODN exposure to embryonic kidneys are included in Fig. 2. A notable decrease was observed in the overall size of day 13 metanephric explants exposed to MT-1-MMP antisense ODN compared with the untreated control (Fig. 2, D vs. A). The ureteric bud branches were rudimentary and had blunted tips (Fig. 2D); the latter are the sites of epithelial:mesenchymal interactions and nascent nephron formation. The nephron population was markedly reduced, and most of the parenchyma was occupied by the loose metanephric mesenchyme. The metanephric explants treated with sense or nonsense ODN had minimal reduction in their overall size or decrease in the population of the developing nephrons (Fig. 2, B and C vs. A). Also, the ureteric bud branches and their tips did not reveal any structural abnormalities. The metanephric treated with MMP-2 antisense ODN had moderate decrease in the size of the explant and nephron population (Fig. 2E). The ureteric bud branches also had undergone dysmorphicogenetic changes, similar to those in explants treated with MT-1-MMP antisense ODN, whereas the explants treated with TIMP-2 antisense ODN exhibited minimal morphological alterations, and only a mild reduction in the nephron population was observed (Fig. 2F). Interestingly, the overall size of the explants seemed to be increased compared with the controls (Fig. 2, F vs. A–C).

The role of MMP-2 activation, MT-1-MMP activity, and TIMP-2 in metanephric development was further investigated by experiments in which the explants were exposed concomitantly either to MT-1-MMP and MMP-2, MT-1-MMP and TIMP-2, or MMP-2 and TIMP-2 antisense ODNs. Concomitant exposure of MT-1-MMP and MMP-2 antisense ODNs resulted in a profound reduction in the size of the explants (Fig. 2, G vs. A–C), suggesting a synergism between the action of these proteases during metanephric development. Concomitant exposure of MT-1-MMP and TIMP-2 (Fig. 2H) or MMP-2 and TIMP-2 (Fig. 2I) antisense ODNs resulted in the partial normalization of the size of the explants and restoration of the morphology of the ureteric bud branches and of the nephron population.
mRNA expression in the antisense-ODN-treated renal explants. To ascertain the transcripional blocking activities of the MT-1-MMP-, MMP-2-, and TIMP-2-specific phosphorothioated antisense ODNs, competitive RT-PCR studies were performed. The method of competitive RT-PCR was chosen to circumvent the difficulties related to the minute amount of mRNA available for Northern blot analysis from antisense-ODN-treated embryonic explants, harvested at day 13 of gestation. In the control (nonsense-ODN-treated group), a linearity in the ratios of wild to mutant MT-1-MMP-DNA could be maintained when plotted against the $10^{-4}$ to $10^{-6}$ serial logarithmic dilutions of the competitive template DNA (Fig. 3A). Within this
range of dilution, the agarose gel electrophoretic bands of wild-type and mutant DNA were distinctly visualized for densitometric analyses and to obtain a ratio. In the samples with lower ($10^{-3}$) and higher ($10^{-7}$) dilutions, the respective gel bands of wild-type and mutant DNA were not discernible, and thus no densitometric readings or ratios could be obtained. In the MT-1-MMP antisense ODN-treated group, a reduction in the MT-1-MMP mRNA expression was observed, since the linearity ratio relationship shifted to a range of $10^{-6}$ to $10^{-8}$ dilutions of the competitive template DNA (Fig. 3A).

For β-actin, no significant shift in the linearity relationship between the two groups (control and antisense), within the range of logarithmic dilutions of the competitive template DNA, was observed (Fig. 3D), indicating no change in its mRNA expression with the antisense ODN treatment. Similarly, no changes in the linearity ratio relationship were observed for MMP-2 (Fig. 3B) and TIMP-2 (Fig. 3C) between the control and experimental groups, suggesting that the steady-state levels of these mRNA transcripts were unaffected and that the effects of MT-1-MMP antisense ODN are specific.
The competitive RT-PCR analyses of the explants treated with MMP-2 antisense ODN revealed a selective reduction in the mRNA expression of MMP-2 (Fig. 4B), whereas that of MT-1-MMP (Fig. 4A), TIMP-2 (Fig. 4C), and of β-actin (Fig. 4D) remained unchanged. Also, the explants treated with TIMP-2 antisense ODN had a selective reduction of TIMP-2 mRNA only (Fig. 5C), and the mRNA expression of MT-1-MMP (Fig. 5A), MMP-2 (Fig. 5B), and of β-actin (Fig. 5D) were unaltered. These results indicate that the antisense ODNs used in this study had specific effects on the expression of their respective mRNA.

Effect of antisense ODN on the MT-1-MMP protein expression. To ascertain the translational blocking activities of the MT-1-MMP-specific phosphorothioated antisense ODNs, immunofluorescence and immunoprecipitation studies were performed. Immunofluorescence studies revealed no significant differences in the immunoreactivity between the untreated control and the explants treated with MT-MMP-2 nonsense ODN (Fig. 6, G vs. A). However, a marked diminution of the anti-MT-1-MMP immunoreactivity was noted in metanephric explants exposed to MT-1-MMP antisense ODN compared with those treated with nonsense ODN (Fig.

Fig. 4. Competitive RT-PCR of MT-1-MMP (A), MMP-2 (B), TIMP-2 (C), and β-actin (D) cDNAs, prepared from nonsense-ODN-treated (control) and MMP-2-antisense-ODN-treated embryonic kidney explants. Serial logarithmic dilutions of mutated competitive DNA template of MT-1-MMP, MMP-2, TIMP-2, and β-actin (see MATERIALS AND METHODS) were coamplified with a fixed amount (1 µl) of first-strand cDNA prepared from metanephric explants, and ratios between the densitometric readings of wild-type and mutant PCR products were plotted on logarithmic scales on the y-axis against the logarithmic dilutions of competitive DNA on the x-axis. A reduction in the amplification of wild-type MMP-2 DNA is observed in the antisense-ODN-treated kidney explants compared with control (B), suggesting a decrease in the mRNA expression. No significant differences are seen in the amplification of MT-1-MMP (A), TIMP-2 (C), and β-actin (D) between the nonsense-ODN-treated (control) and antisense-ODN-treated groups.
6, D vs. A). No significant decrease of anti-MMP-2 immunoreactivity was observed with MT-1-MMP antisense ODN treatment (Fig. 6, E vs. B). Similarly, no notable reduction in the TIMP-2 immunoreactivity was observed (Fig. 6, F vs. C). The treatment with MMP-2- or TIMP-2 antisense ODN resulted in a marked decrease in the immunoreactivity of MMP-2 (Fig. 6H) and TIMP-2 (Fig. 6I), respectively. No significant decrease in the immunoreactivity was observed in the explants treated with MMP-2- or TIMP-2-nonsense ODNs compared with the untreated embryonic explants (data not shown). These findings suggest that the translational blockade is specific, and the antisense-ODN-induced translational blockade effect was further confirmed by immunoprecipitation of the extracts of $^{35}$S-methionine-labeled metanephric explants followed by 10% SDS-PAGE and autoradiography. The autoradiograms revealed a notable reduction in the intensity of the ~63-kDa band in the explants treated with antisense ODN compared with the control (Fig. 7A, lane 3 (AS) vs. lane 1 (CON)). The intensity of the autoradiographic band of the explants treated with nonsense ODN was similar to that of the control (Fig. 7A, lane 2 (NS) vs. lane 1 (CON)).

Effect of MT-1-MMP antisense ODN on the activation of MMP-2. Zymographic studies were carried out to
assess whether the downregulation of MT-1-MMP with the antisense ODN influenced the conversion of pro-MMP-2 into its active 62-kDa form. The zymograms revealed that the antisense-ODN-induced translational blockade of MT-1-MMP effectively inhibited the conversion of pro-MMP-2 to its active form (Fig. 7B).

The zymogram of the metanephric explants treated for 4 days with nonsense ODN revealed a major 72-kDa band of pro-MMP-2 and ~64- and ~62-kDa bands of intermediate and fully active forms of MMP-2, respectively [Fig. 7B, lane 2 (4d-NS)]. Although the band of intermediate form of MMP-2 was not readily discernible after 1-day treatment with MT-1-MMP antisense ODN, the band of fully active form was clearly visualized [Fig. 7B, lane 1 (1d-AS)]. Interestingly, the intensity of the bands corresponding to the intermediate
and fully active (~62 kDa) forms of MMP-2 was remarkably reduced in the explants treated with antisense ODN for 4 days in the organ culture [Fig. 7B, lane 3 (4d-AS)]. In addition, the intensity of pro-MMP-2 band was much greater in the 4-day-treated antisense ODN group compared with the explants exposed to nonsense ODN for 4 days or antisense ODN for 1 day [Fig. 7B, lane 3 (4d-AS) vs. lane 2 (4d-NS) and lane 1 (1d-AS)]. Finally, a diffuse band with molecular mass greater than 72 kDa was also observed in the zymograms of explants treated with antisense and sense ODNs, and this may correspond to other MMPs, e.g., MMP-8 or MMP-9.

**DISCUSSION**

The interest in the role of MMPs in embryogenesis has been limited, and there are few reports available in the literature describing their role in mammalian development. Their role in early embryonic development has been suggested, since MMP-3 and TIMP-1 in peri-implantation embryos and TIMP-1 and -3 in decidual tissues are expressed (2, 7). In later stages of embryogenesis, the role of MMPs in organogenesis becomes quite relevant, since the growth factors and receptors that greatly influence the biology of development also modulate the expression of MMPs (1). Data relevant to organogenesis have been derived from studies of transgenic mice in adult life, where an overexpression of MMP-3 resulted in an accentuated branching morphogenesis of the mammary gland (73, 77, 87). The preliminary results of our studies indicating the expression of MT-1-MMP in the embryonic metanephros provided us with an impetus to conduct this investigation as to the role of MT-1-MMP in organ development and relationship with the activation of pro-MMP-2 and the expression of MMP-2 and its inhibitor, TIMP-2.

This contention that MT-1-MMP may be involved in the organogenesis of various tissues is supported by the demonstration that it is expressed in the embryonic metanephros and that it is also developmentally regulated (Fig. 1A). The fact that mRNA expression seems to be high during the mid to late stages of gestation, the period when organogenesis is at its peak, lends further support to this notion. Interestingly, the expression of MMP-2 parallels the expression of MT-1-MMP (52), which suggests that they may have a conjoint role in...
the morphogenesis of the kidney. Such a parallel coexpression of MT-1-MMP and MMP-2 has also been observed in ossifying tissues during mouse embryogenesis (31). Furthermore, the fact that the MT-1-MMP mRNA expression is spatially restricted to the epithelial components of the kidney, i.e., the ureteric bud branches, while that of the MMP-2 is concentrated in the surrounding mesenchyme (59, 76), would imply their potential role in epithelial:mesenchymal interactions that are prevalent during fetal development. Such paracrine/juxtacrine interactions may indeed be feasible in view of the previously published data that MT-1-MMP and MMP-2 proteins are coexpressed in the epithelia of ureteric bud branches and that, like mRNA expression, the protein expression is also developmentally regulated (52). The immunolocalization of MMP-2 to the ureteric bud may be related to its binding to the plasmalemmal MT-1-MMP after it being secreted out from mesenchymal cells of the metanephros. Such a translocation of MMP-2, i.e., from stromal to epithelial cells, has been suggested by in situ hybridization studies in various human neoplasms as well (55, 57). It is believed that such a translocation may facilitate the invasion and local metastasis of epithelial tumors into the surrounding stroma upon activation of MMP-2 by MT-1-MMP (32). In this regard, it is interesting to note that an increased expression of both MT-1-MMP and MMP-2 has been observed in human neoplasms with metastatic potential (21, 39). The inhibitor of MMP-2, i.e., TIMP-2, which binds to MMP-2 with 1:1 molar stoichiometry (10), may limit the invasion of cancer cells into the stroma, since an increased expression of TIMP-2 has been reported to reduce the metastatic potential of the invasive tumor cells (18, 71). Here, it is worth mentioning that TIMP-2 expression is also developmentally regulated in the embryonic metanephros (52), and its maximal expression, however, is slightly delayed with respect to MT-1-MMP and MMP-2, which would suggest that the latter is involved in remodeling of the metanephros during the postnatal life. Thus, at present, it appears that the trimacromolecular complex of MT-1-MMP:MMP-2:TIMP-2 (27, 49, 72) may be essential for the cell-mediated activation of pro-MMP-2 and participation in processes that regulate epithelial:mesenchymal interactions, including tumor invasion and embryogenesis.

The role of secretory type of MMPs, described as collagenases in the older literature, in organotypic epithelial:mesenchymal interactions was proposed three decades ago by Grobstein and Cohen (23) and Wessells and Cohen (85). They postulated that a concentration gradient of ECM proteins, generated by the action of MMPs, is essential to the cleft formation and lobulogenesis of glandular tissues. Direct evidence implicating a differential gradient of collagen fragments initiating cleft formation and lobulogenesis was provided by studies in which treatment with bacterial collagenase perturbed the branching morphogenesis of salivary glands (20, 24, 51). In contrast, collagenase inhibitors stimulated branching morphogenesis in these assays. Further support for the role of secreted-type of MMPs in morphogenesis of glandular tissues was obtained by transgenic studies of stromelysin-1 function in mammary gland morphogenesis, as mentioned previously (73, 87).

The present study describes the role of MT-1-MMP in organogenesis in the context of paracrine/juxtacrine epithelial:mesenchymal interactions and the activation/inhibition cascade of MT-1-MMP, MMP-2, and TIMP-2, using antisense ODNs. Antisense ODNs have been successfully used to study various developmental processes, e.g., neural crest migration (36) and metanephric organogenesis (40, 80). In these studies, it is critical to the successful interpretation of the results that the specificity and effectiveness of the antisense ODN be demonstrated and maintained. As reported in this study, we monitored the specificity and effects of the various antisense ODNs by S1 nuclease assay (Fig. 1B). In addition, competitive RT-PCR analyses and translational blockade experiments using specific antibodies to detect the translation products were carried out. The MT-1-MMP antisense ODN induced marked dysmorphogenesis of the metanephros (Fig. 2D). The changes in the metanephros included an overall decrease in its size, reduced population of the nascent nephrons, and disorganization of the ureteric bud branches and blunting of their tips. The latter anatomical deformity, i.e., loss of acuteness of the tips, is believed to interfere in the nephrogenesis by perturbing the epithelial:mesenchymal interactions. Such changes have also been observed with the disruption of paracrine/juxtacrine interactions, where the ligand or a growth factor is expressed in the metanephric mesenchyme and its receptor or protooncogene, serving as a receptor, is expressed in ureteric bud epithelia (40, 80). In an analogous manner, it is conceivable that MT-1-MMP antisense ODN treatment may have disrupted the epithelial:mesenchymal interactions, which led to the formation of relatively few nephrons. Similarly, antisense ODN of MMP-2 also induced moderate dysmorphogenetic changes (Fig. 2E), suggesting that the MT-1-MMP-mediated conversion of pro-MMP-2 to an active protease may be essential for metanephrogenesis (see below). The results of MMP-2 experiments differ from those reported in the literature (38), in which anti-MMP-2 antibody was found to have no effect on metanephrogenesis. The differences may be related to the specificity of antibody (anti-human vs. anti-mouse) and the stage at which metanephric were procured for the morphogenetic experiments, as well as the poor accessibility of MMP-2 in the tissue microenvironment, i.e., cell surface localization. It should be noted that the effects of antisense ODNs were highly specific, since the treatment with TIMP-2 antisense ODN did not reduce the size of the metanephros; rather it was somewhat increased, and the ureteric bud branches retained their normal iterations (Fig. 2F), an effect opposite of MT-1-MMP and MMP-2. Furthermore, the fact that TIMP-2 antisense ODN caused a partial normalization of the morphology in explants treated with MT-1-MMP/MMP-2 antisense ODN (Fig. 2, H and I) strongly indicates a high degree of specificity of the antisense
MMP-1 mRNA expression coinciding with the inhibitory effect of TIMP-2 in the embryonic development of the kidney. The selectivity of the effects of MT-1-MMP, MMP-2 and TIMP-2 antisense ODNs were rigorously examined by RT-PCR analyses, where minimal alterations in the gene expression of the other components of the trimacromolecular complex were observed (Figs. 3–5). Similarly, the MT-1-MMP antisense ODN-treated explants revealed a drastic reduction in the immunoreactivity with the anti-MT-1-MMP antibodies (Fig. 6D), whereas a mild decrease in the immunoreactivity of anti-MMP-2 and -TIMP-2 was also observed (Fig. 6, E and F). In this regard, recent studies suggest that the bulk of the secreted MMP-2 and TIMP-2 are soluble and a small fraction is localized on the cell surface via the formation of a trimacromolecular complex with MT-1-MMP (27, 49, 72). It is probable that the mild decrease of protein localization may be due to the loss of plasmalemmal MT-1-MMP, which disrupted the binding of secretory type MMP-2 and TIMP-2 to the ureteric bud epithelia. In addition to the mild decreased anti-MMP-2 immunoreactivity, gene disruption or translational blockade of MT-1-MMP may have also contributed to a loss in the activation of pro-MMP-2 to MMP-2, which then resulted in dysmorphogenesis of the embryonic kidney. This may be the case, since the results of zymographic and morphological studies indicated that the gene disruption or translational blockade (Figs. 3A and 7A) of MT-1-MMP led to a failure in the activation of pro-MMP-2 to MMP-2 (Fig. 7B). Taken together, the results of zymographic and morphological studies indicate that the gene disruption of MMPs, i.e., MT-1-MMP and MMP-2, induced dysmorphogenesis of embryonic metanephros, which may be due to an independent or interdependent loss of their activities. Moreover, since TIMP-2 antisense ODN has somewhat of an opposite effect, it seems that TIMP-2, by downregulating the activity of MMP-2, serves as an integral participant in organogenesis. To address other possible mechanisms by which the MMPs influence the branching morphogenesis of the kidney, Nigam and his colleagues (60, 62) utilized the inner medullary collecting duct cells in type I collagen gel culture system. They were able to document a rise or fall in the MMP-1 mRNA expression coinciding with the inhibition or stimulation of branching morphogenesis under the influence of transforming growth factor-β (TGF-β) or ligands of epidermal growth factor receptor, such as TGF-α. The fact that TGF-α and TGF-β regulate the expression of matrix proteins would imply that the biology of growth factors and their receptors and of the ECM-related macromolecules, i.e., MMPs, is intricately linked during the process of metanephric development.

In summary, the developmentally regulated mRNA expression and the antisense ODN data of this investigation lend a reasonable support to the contention that MT-1-MMP activation of pro-MMP-2 and TIMP-2 modulation of these protease activities are essential for normal ECM remodeling processes and epithelial-mesenchymal interactions to sustain organogenesis of the embryonic metanephros.

We are thankful to Dr. William G. Stetler-Stevenson for providing us with anti-MMP-2 and anti-TIMP-2 antibodies. This investigation was supported by National Institute of Diabetes and Digestive and Kidney Diseases Grant DK-28492.

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REFERENCES

METALLOPROTEINASES IN DEVELOPMENT


Howlett, A. R., and M. J. Bissell.


Lelongt, B., G. Trugman, G. Murphy, and P. M. Ronco.


