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Gene transfer of metalloproteinase transin induces aberrant behavior of cultured mesangial cells

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Gene transfer of metalloproteinase transin induces aberrant behavior of cultured mesangial cells. The aim of the present study is to clarify whether the cellular expression of a matrix-degrading metalloproteinase, transin, alters the behavior of cultured mesangial cells (MCs). The cDNA encoding rat transin was introduced into rat MCs and transcribed under the control of a Rous sarcoma virus promoter. The resulting transfectants were then investigated for cell shape, migration, proliferation, and expression of genes associated with matrix metabolism. Northern blot analysis routinely detected the transin transcript in two separate transfectants, MeTRN2 and MeTRN5. Transin expression was strong in MeTRN2, moderate in MeTRN5, but absent in mock transfectants. Immunoblot analysis revealed that these transin transfectants synthesized 59 and 62 kDa molecules, which correspond to transin gene products. Casein digestion assay detected enhanced proteolytic activity in MeTRN2 and MeTRN5. Microscopically, the transfected cells were somewhat elongated with accentuated margins compared with mock transfectants. [^{3}H]-thymidine uptake studies revealed accelerated growth of the transfectants on a plastic substratum as well as within gel matrix. The migration of the transfectants into gel matrix was also significantly enhanced compared with that of mock transfectants. No obvious alteration, however, was found in transcripts of procollagen $\alpha 1$(IV), laminin B_2, or the metalloproteinase inhibitor TIMP. We hypothesize that the metalloproteinase transin has a potential for affecting the behavior of MCs and contributing to the pathogenesis of glomerular injury.

Extracellular matrix (ECM) regulates the behavior of many cell types and, thereby, contributes to organ development, tissue repair, and maintenance of normal tissue structure and function [1]. In the renal glomeruli, ECM may also play an important role in the regulation of cell behavior and the maintenance of glomerular architecture. We previously reported that the three-dimensional environment of basement membrane-type matrix inhibits elongation, proliferation, and migration of mesangial cells (MCs) *in vitro* [2]. Normal mesangial matrix may then have an intrinsic potential for repressing the abnormal behavior of MCs. However, the mesangial matrix is degraded by matrix proteinases, especially by metalloproteinases. The resulting failure of ECM to perform its regulatory function could permit deviant behavior by MCs. Several lines of evidence support this hypothesis: (i) Various matrix-degrading proteinases can modulate the shape and growth of cells *in vitro* [3–6]. (ii) Habu venom, a member of the metalloproteinase family, can induce mesangiolysis followed by marked MC proliferation *in vivo* [7]. (iii) Metalloproteinase expression in the mesangium is closely associated with MC proliferation in an experimental model of glomerulonephritis [8]. Therefore, metalloproteinases may play a pathological role not only in the abnormal turnover of mesangial matrix, but also in the aberrant behavior of MCs.

Our previous report demonstrated that cultured rat MCs stimulated by IL-1, one of major players involved in the pathogenesis of glomerular diseases [9–12], abundantly expressed transin (rat stromelysin) [13]. Transin is a metalloproteinase of broad substrate-specificity, capable of degrading collagens, glycoproteins, and proteoglycans [14], all of which are major constituents of the mesangial matrix and glomerular basement membrane (GBM). Indeed, Bejarano and colleagues [15] reported that stromelysin degrades glomerular ECM effectively *in vitro*.

Therefore, we undertook the present study to clarify how the multipotent matrix-degrader, transin, when expressed in a dysregulated manner, affects the behavior of MCs. Using *in vitro* gene transfer technology, we established clones of MCs stably expressing transin, and assessed their behavioral characteristics within ECM as well as on a plastic substratum.

Methods

Experimental design

A full-length cDNA of rat transin was subcloned into a eukaryotic expression vector, pRc/RSV, and transfected into rat MCs by a calcium-phosphate method. Stable transfectants were selected in the presence of a neomycin analog G418, and their expression of the transin transcript and of its product was confirmed by RNA blot analysis or immunoblot analysis. Proteolytic activity of transfectants was also examined using a casein digestion assay. Then, we assessed these cells' changes in shape, proliferation, migration, and expression of genes associated with matrix metabolism by the methods described below.

Mesangial cells

Rat mesangial cells (MCs) from isolated glomeruli of adult Sprague-Dawley rats were cultured as previously reported [16].

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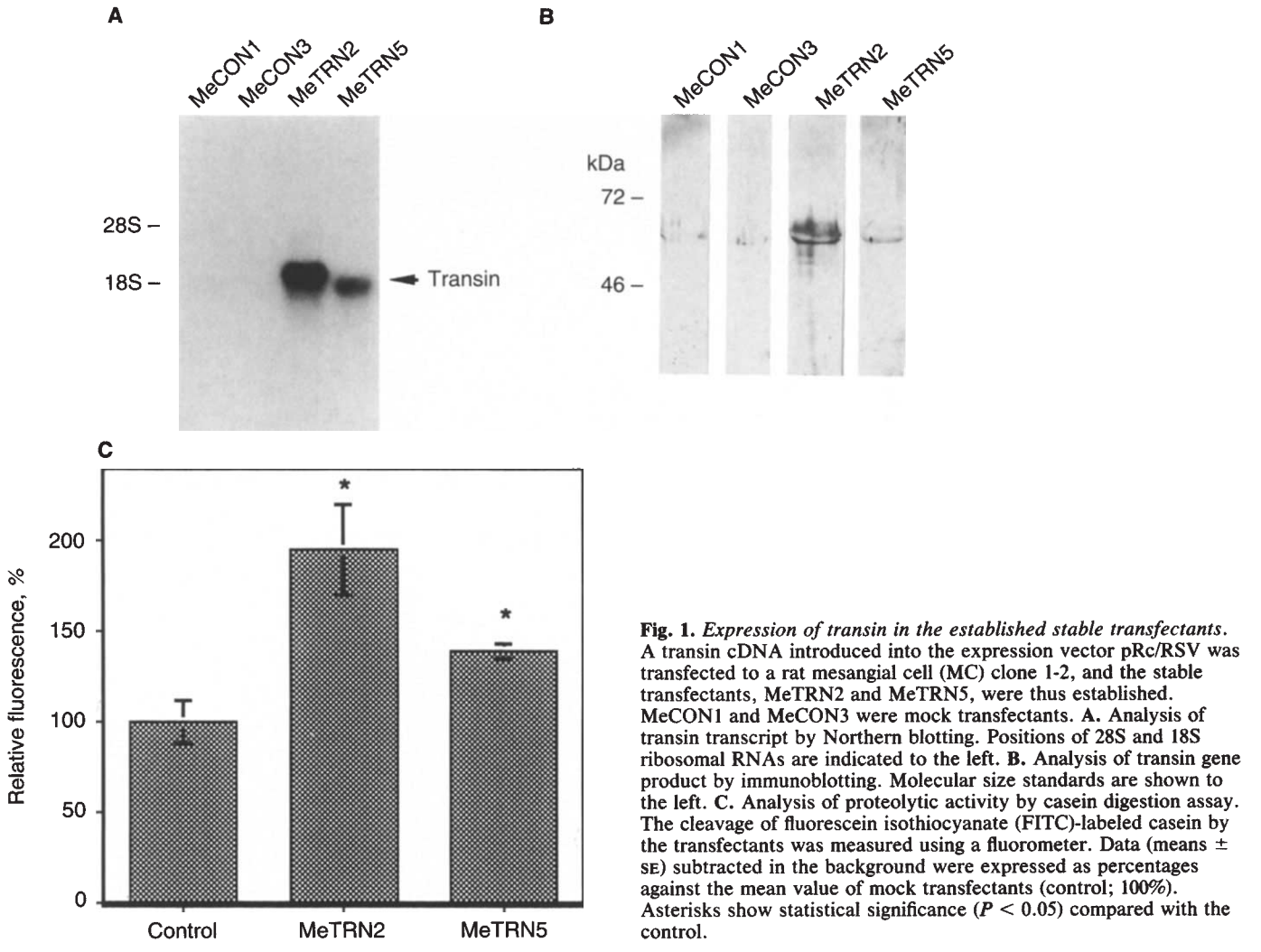

Fig. 1. *Expression of transin in the established stable transfectants.* A transin cDNA introduced into the expression vector pRc/RSV was transfected to a rat mesangial cell (MC) clone 1-2, and the stable transfectants, MeTRN2 and MeTRN5, were thus established. MeCON1 and MeCON3 were mock transfectants. **A.** Analysis of transin transcript by Northern blotting. Positions of 28S and 18S ribosomal RNAs are indicated to the left. **B.** Analysis of transin gene product by immunoblotting. Molecular size standards are shown to the left. **C.** Analysis of proteolytic activity by casein digestion assay. The cleavage of fluorescein isothiocyanate (FITC)-labeled casein by the transfectants was measured using a fluorometer. Data (means ± SE) subtracted in the background were expressed as percentages against the mean value of mock transfectants (control; 100%). Asterisks show statistical significance ($P < 0.05$) compared with the control.

Since cultured MCs may contain heterogeneous populations, we used an MC clone specifically for the transfection study. Clone 1-2 was established by limiting dilution from the cultured rat MCs. This clone was confirmed as MCs by positive staining for actin and desmin, and negative staining for cytokeratin and factor VIII [16]. The MCs were maintained and all assays were performed in Dulbecco's modified Eagle's medium (DMEM; GIBCO) supplemented with 100 U/ml penicillin, 100 μg/ml streptomycin, and 5% fetal calf serum (FCS; Cell Culture Laboratories).

Construction of recombinant plasmids and establishment of stable transfectants

The *Eco* RI fragment of a full-length rat transin cDNA [17] (a gift from Dr. L. M. Matrisian, Vanderbilt University, Nashville, Tennessee, USA) was blunt-ended and introduced into a blunt-ended *Hind* III site of pRc/RSV (Invitrogen) in a sense orientation (pRc/RSVTR). A recombinant plasmid with an insert of an anti-sense orientation (pRc/RSVATR) was prepared as a control. All the plasmids were purified twice by CsCl gradient centrifugation and used for transfection.

By using a modified calcium-phosphate method [18], clone 1-2 was transfected with pRc/RSVTR, pRc/RSVATR, or pRc/RSV. Stable transfectants were selected in the presence of G418 (Sigma; 0.75 mg/ml). Five clones examined in this study were as follows: two sense transin transfectants, MeTRN2 and MeTRN5; one anti-sense transin transfectant, MeATR2; and two mock transfectants, MeCON1 and MeCON3. These transfectants were assessed during the period between the 10th and 20th passages.

Assessment of transin expression

Northern blot analysis. To assess transin gene expression in transfectants, total RNA was extracted by a single-step method [19], and Northern blot analysis was performed as described before [20]. The RNA samples (10 μg/lane) were electrophoresed on 1.2% agarose gels containing 10% formaldehyde, and transferred onto nitrocellulose membranes (Schleicher & Schuell, FRG). For the hybridization, *Eco* RI fragment of transin cDNA was labeled with ^{32}P-dCTP using a random priming method [21]. These membranes were hybridized with probes at 65°C for 16 hours in a solution containing 4× SSC (600 mM sodium chloride, 60 mM sodium citrate), 5× Denhardt's solution, 10% dextran sulfate, and 100 μg/ml herring sperm

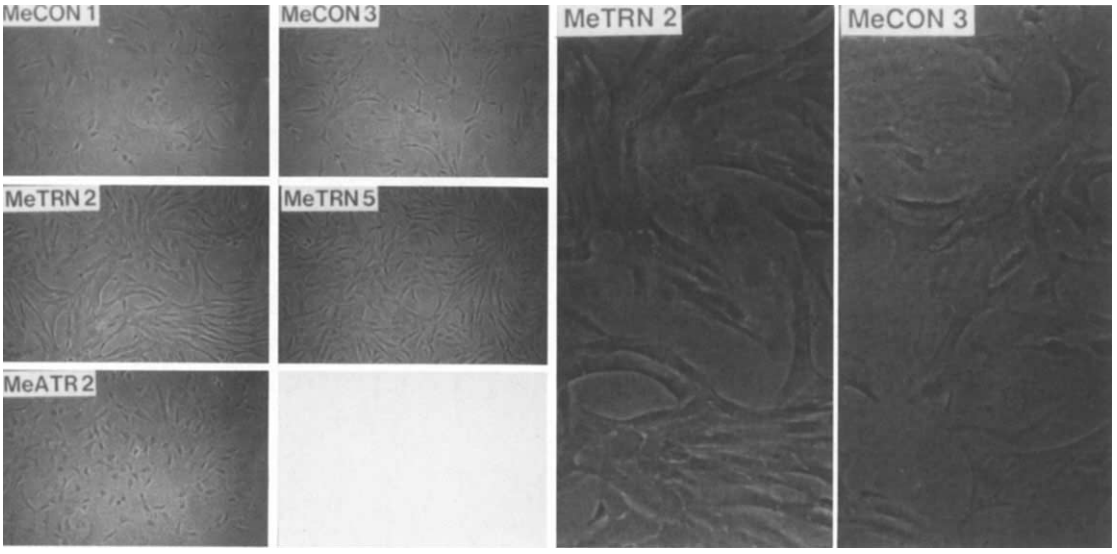

Fig. 2. *Effect of transin expression on the shape of MCs.* Each transfectant was cultured on a plastic substratum for three days, and morphological study was performed by phase-contrast microscopy. MeATR2 was a transin transfectant in the anti-sense orientation.

DNA. Afterward, the hybridized membranes were washed four times in 4× SSC/0.1% SDS at 50°C, and exposed to Kodak XAR film with an intensifying screen at −70°C.

Immunoblot analysis. To detect the translational product of the transin cDNA, immunoblot analysis was performed by an immunoperoxidase method. Each confluent transfectant (1×10^6 cells) was washed with PBS and harvested by using a rubber policeman. Cell pellets were suspended in 200 μl lysis buffer [2% digitonin, 0.5 M sucrose, 2 mM EDTA(2Na), 20 mM PBS, pH 7.2, 0.02% sodium azide], and incubated on ice for 15 minutes. After centrifugation, an equal volume of 2 × SDS gel-loading buffer (100 mM Tris-HCl, pH 6.8, 200 mM dithiothreitol, 4% SDS, 0.2% bromophenol blue, 20% glycerol) was added to each supernatant, and 10 μl of sample was separated on a 15% SDS-polyacrylamide gel. Proteins were electrotransferred to nitrocellulose membranes (Schleicher & Schuell). The filters were blocked in 10% nonfat milk, incubated with sheep anti-human matrix metalloproteinase (MMP)-3 antiserum (a gift from Dr. Y. Okada, Kanazawa University, Tokyo, Japan) diluted 1:500 in PBS containing 1% milk at 37°C for one hour, washed with PBS, and incubated with peroxidase-conjugated anti-sheep immunoglobulins (DAKO, Denmark) diluted 1:200 in PBS. The enzymatic reaction was performed in 0.1 M Tris-HCl containing 0.2 mg/ml of diaminobenzidine (Sigma) and 0.02% H_2O_2.

Casein digestion assay. Casein is commonly used to measure stromelysin activity because this substrate is minimally influenced by ubiquitous metalloproteinases, type IV collagenases. To examine the proteolytic activity of the stable transfectants, we directly applied fluorescein isothiocyanate (FITC)-labeled casein (Sigma) [22] to the culture of the transfectants. FITC-casein was dissolved in 5% FCS/DMEM at a concentration of 50 μg/ml, sterilized using 0.2 μm filter (Corning), and 1 ml of its aliquot was added to each confluent culture of mock or transin transfectants in 6-well plates. Wells without cells but containing 1 ml casein media were used for assessing background. After incubating for 18 hours at 37°C, 1 ml of 10% trichloroacetic acid (TCA) was added to each medium to remove uncleaved casein. The TCA-insoluble protein was sedimented by centrifugation, and a 150 μl aliquot of each supernatant was mixed with an equal volume of 1 M Tris. Fluorescence was determined by a fluorometer (Fluoroskan II; Flow Laboratories) using an excitation wavelength of 485 nm and an emission wavelength 538 nm. In transin transfectants, the individual values subtracted in the background were compared with the values for mock transfectants. The assay was performed in quadruplicate.

Assessment of cell behavior

Cell shape. Each transfectant (5×10^4 cells) was cultured on a 35 mm plastic plate (Nunc Inc.) for three days. Cell shape was examined in a phase-contrast microscope.

Growth. (i) Growth on a plastic substratum. Each transfectant was suspended in 5% FCS/DMEM and dispensed into 96-well culture plates at a density of 1×10^3 cells per well. After three days' culture, [^{3}H]-thymidine (0.1 μCi per well) was added to each well. Two days later, cells were harvested, and incorporation of [^{3}H]-thymidine was measured by a liquid scintillation counter as an indicator of cell growth. The study was performed in quadruplicate.

(ii) Growth in gel matrix. Each transfectant was placed into a mixed gel composed of 25% Matrigel™ (Collaborative Research) and 75% type I collagen gel (derived from rat tail tendon [16]) then assayed as reported previously [2]. In brief, each well of a 96-well culture plate was coated with 50 μl of mixed gel solution without cells. After the gel formed, 50 μl of mixed gel solution containing 2×10^4 cells was overlaid on the basal layer. After the second gel formed, 100 μl of 10% FCS/DMEM were dispensed into each well to adjust the final concentration to 5% FCS. Cells were precultured for three days, then pulsed with [^{3}H]-thymidine, 0.1 μCi per well. Three days later, the gel matrix containing cells was harvested with extensive washing, and the incorporation of [^{3}H]-thymidine was assessed. All experiments done were in quadruplicate.

Migration. To evaluate cell migration into gel matrix, we used an assay system established previously [2]. A sterilized polypropylene ring was attached to the center of each 35 mm culture dish, separating the dish into two compartments. After 5 μl of Matrigel was placed in the center of the inner area and gelled, each transfectant was suspended in 5% FCS/DMEM at a concentration of 1×10^5 cells/ml, plated into the outer area, and incubated for two days. After exchanging the medium in each outer area, the ring was removed to permit cell migration toward the central gel area. Twelve days later, the extent of cell migration into the gel area was assessed by phase-contrast microscopy. To evaluate this migration quantitatively, four axes at 45° angles to one another were randomly chosen on each gel, and eight migratory distances from gel edge to migration front were measured along each axis. The means of these values were used as an index for migration activity. The assay was performed in quadruplicate.

Expression of genes associated with matrix metabolism. To assess the effect of transin introduction on the expression of other genes associated with matrix metabolism, transcripts of procollagen α1(IV), laminin B_2, transin, and tissue inhibitor of metalloproteinases (TIMP) were investigated by Northern blot analysis. The cDNA probes, mouse procollagen α1 (IV) and mouse laminin B_2 cDNAs were gifts from Dr. Y. Yamada (NIH), and human TIMP cDNA from Dr. M. Naruto (Toray Industries, Japan).

Statistical analysis

Data were expressed as means ± SE. Statistical analysis was performed with the Mann-Whitney test.

Results

Expression of transin in the transfectants

To assess whether a transin gene introduced into rat MCs was successfully transcribed, we first examined the transin transcript in two transfectants, MeTRN2 and 5, established for this study. Northern blot analysis revealed that both transfectants stably expressed 2 kb of transin mRNA; expression was higher in MeTRN2 but still moderate in MeTRN5 (Fig. 1A). No obvious expression of transin was detected in mock transfectants, MeCON1 and MeCON3.

We then used immunoblot analysis to ascertain whether the transcribed RNA was translated into protein in these transfectants. Stromelysin, a human homologue of transin, is secreted from cells in two latent forms, a molecule of 57 kDa and another of 60 kDa; these unglycosylated and glycosylated species are converted to an active form in the extracellular space [23]. Using anti-MMP-3 antibody, we detected 59 and 62 kDa molecules in the transin transfectants (Fig. 1B), a size that approximates the latent form of transin [24]. These two molecules were not detected in our mock transfectants but were markedly induced in them by IL-1β (data not shown), a stimulator of transin in MCs [13]. Additionally, the amount of transin detected in the transfectants was closely associated with mRNA level of transin (Fig. 1 A, B).

To determine whether the transin transfectants exhibit enhanced proteolytic activity, the cleavage of FITC-casein was directly examined in culture. The casein digestion assay detected increased proteolytic activity in MeTRN2 (195 ± 25%) and in MeTRN5 (139 ± 2%) compared with the mean value of mock transfectants (Fig. 1C). These findings showed that the introduced transin cDNA was successfully transcribed, translated, and converted to an active enzyme *in vitro*.

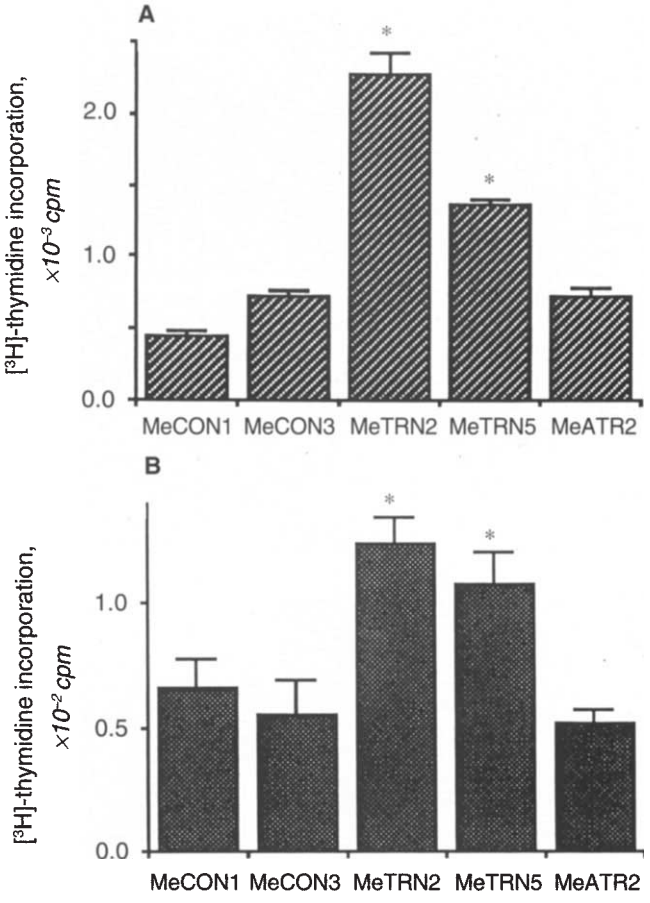

Fig. 3. *Effect of transin expression on the proliferation of MCs.* Growth activity of each transfectant on a plastic substratum **(A)** or in gel matrix composed of 25% Matrigel and 75% type I collagen gel **(B)** was assessed by the incorporation of [^{3}H]-thymidine. Data are expressed as means ± SE. Asterisks show statistical significance ($P < 0.05$) compared with three different controls.

Behavioral alteration in the transin transfectants

To investigate the phenotypic changes in transin transfectants, we examined their shapes in a phase-contrast microscope. In this experiment, identical numbers of transfected cells were plated on a plastic substratum in the same culture conditions. Both transin transfectants, MeTRN2 and MeTRN5, had elongated shapes with accentuated cell margins (Fig. 2). In contrast, the mock transfectants MeCON1 and MeCON3, and the anti-sense gene transfectant MeATR2, showed a flattened shape with vague cell margins.

Since a heightened proliferative response is one of the representative pathological abnormalities of MCs observed in glomerular diseases, we next investigated whether and how the cell replication was affected by transin overexpression. On a plastic substratum, proliferative activity was accelerated in transin transfectants compared with controls (368 ± 23% in MeTRN2, and 220 ± 4% in MeTRN5 vs. the mean value of

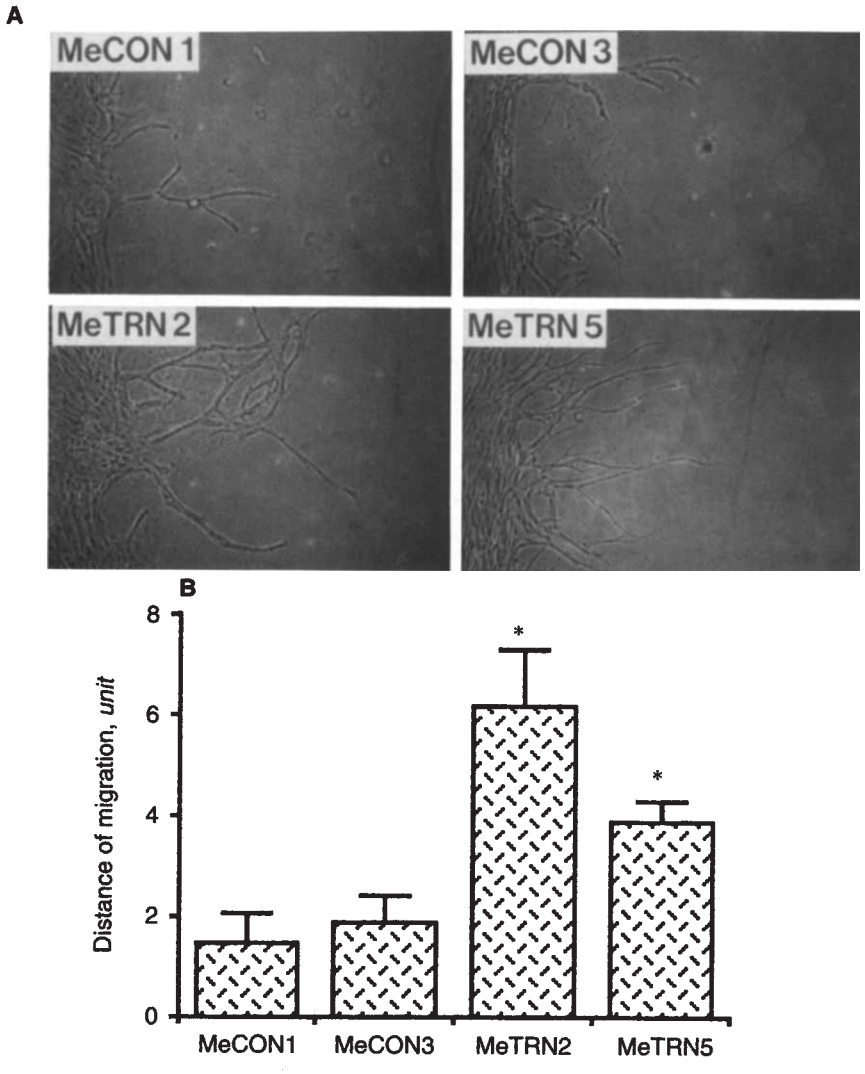

Fig. 4. *Effect of transin expression on the migration of MCs.* Migration activity into Matrigel was examined in each transfectant. **A.** Phase-contrast microscopic analysis. **B.** The degree of cell migration in each transfectant was quantitatively assessed. Data are expressed as means ± SE. Asterisks show statistical significance ($P < 0.05$) compared with both controls.

three controls; Fig. 3A). Within a matrix of 25% Matrigel and 75% collagen gel, the growth of transin transfectants was also enhanced compared with that of the mock or anti-sense transfectants (215 ± 36% in MeTRN2, and 187 ± 43% in MeTRN5 vs. the mean of controls; Fig. 3B). Additionally, the degree of this accelerated growth in MeTRN2 and MeTRN5 correlated closely with the amount of transin expressed, as shown in Figure 1.

Another important abnormality of MC behavior in glomerular disease is the migration of MCs along the GBM, which results in "mesangial interposition." We, therefore, tested whether the migration of MCs was significantly enhanced in transin transfectants. The transfectants showed accelerated migration into Matrigel, 367 ± 68% in MeTRN2 and 231 ± 26% in MeTRN5 against the mean value of mock transfectants (Fig. 4A). The degree of the migration activity also correlated with the amount of transin expressed (Fig. 4B).

Since abnormal matrix turnover in MCs also plays a critical part in the pathogenesis of glomerular diseases, we investigated the dynamics of the molecules involved in matrix metabolism in transin transfectants. Northern blot analysis detected no obvious differences in the transcriptional level of procollagen α1(IV), laminin B_2, or TIMP between transin transfectants and mock transfectants (Fig. 5). These findings suggest that the gene expression in transin transfectants was, as far as we investigated, modified solely by transin overexpression, which had no obvious effect on the transcripts of matrix components or their degradation inhibitor, TIMP.

Discussion

Gene transfer technology is one of the most powerful strategies available for elucidating unknown pathophysiological functions of genes *in vivo* and *in vitro*. However, this technology has rarely been applied in research on glomerular pathophysiology. In the present study, we introduced the metalloproteinase transin cDNA into cultured MCs using a eukaryotic expression vector, pRc/RSV. Stable transfectants established in this way expressed 2 kb of a transin transcript and two major forms of transin, 59 and 62 kDa, respectively as described here. These findings suggest that LTR derived from Rous sarcoma virus works stably and efficiently to transcribe the exogenous gene in MCs. This is further supported by the report of Thomas and

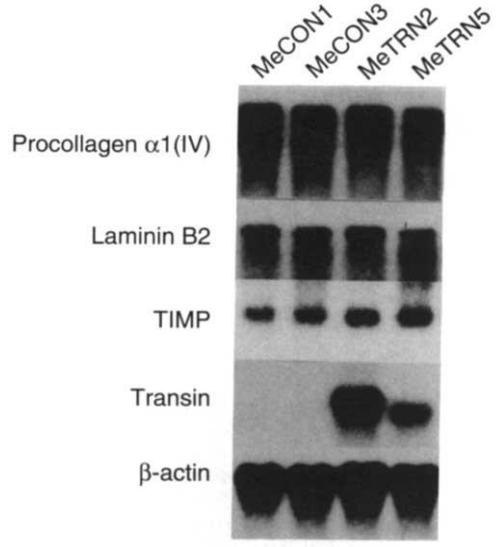

Fig. 5. *Effect of transin introduction on the expression of genes associated with matrix metabolism in MCs.* Steady-state mRNA level of procollagen α1(IV), laminin B_2, and tissue inhibitor of metalloproteinases (TIMP) was examined by Northern blot analysis. Expression of β-actin gene was used as a loading control.

Dunn [25]. The 59 and 62 kDa molecules we detected in transfectants apparently corresponded to the latent species of transin, indicating that the transfected transin gene had been transcribed in MCs in a dysregulated manner. Since the amount of transin in our transfectants approximated the mRNA level in transin, production may be regulated at the transcriptional level. Additionally, casein digestion assay demonstrated that this recombinant proteinase can be converted to an active enzyme in culture.

Having successfully established these transfectants, we examined how their expression of transin, a multipotential matrix-degrader, altered their behavior. The data presented here suggest that the expression of transin induces proliferation and enhanced migration of MCs, as well as altering them morphologically compared with control MCs. Deduced from the fact that transin is markedly induced in MCs stimulated by the inflammatory cytokine IL-1β [13], this molecule may play a role in altering the behavior of MCs as well as destructing ECM and, thereby, contributing to the mesangial damage during glomerular diseases. Transin could modulate the behavior of MCs via several mechanisms. (i) ECM may become degraded and unable to exert its regulatory effect, thus permitting the aberrant behavior of MCs. (ii) Transin can degrade ECM and release several matrix fragments [15], some of which could act as biological modulators for MCs and alter their behavior [26]. (iii) Transin may alter the cell-to-cell or the cell-to-matrix interaction by affecting the turnover of adhesion molecules, as reported [27]. (iv) Growth factors bound to matrix components and stored in ECM [28] could be released as transin degrades matrix components, facilitating their access to cell surface receptors [29]. Furthermore, transin might be involved in the conversion of a latent growth factor into the active form, as suggested for other metalloenzymes [30]. (v) Since transin helps to activate other matrix proteinases [31], such an interaction may have enhanced the biological activity of the transin observed here. (vi) The effect of transin on MCs could result from acting as a growth factor, like the other proteolytic enzymes, urokinase and thrombin [32, 33].

Several reports have suggested that the expression of transin/stromelysin relates to phenotypic "transformation" of some cells including tumor cells, synoviocytes, and vascular smooth muscle cells [24, 34, 35]. These "transformed" cells play a pivotal role in the pathogenesis of tumor invasion/metastasis, rheumatoid arthritis, and atherosclerosis. In glomerular injury, the involvement of metalloproteinases has been also suggested, especially in the light of ECM damage and proteinuria [8, 36, 37]. Still unknown, however, is their contribution to the behavioral abnormality of MCs including the proliferation and the interposition, both of which are frequently observed in affected glomeruli. Our present data offer insight into metalloproteinases' potential for mediating the phenotypic "transformation" of MCs during disease and for remodeling glomeruli. We hypothesize here that the metalloproteinases may be involved not only in abnormal degradation of the ECM, but also in the aberrant behavior of MCs, both of which can contribute to the pathogenesis of glomerular injury.

Acknowledgments

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