Role of Mast Cell Tryptase in Renal Interstitial Fibrosis

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Abstract. Renal interstitial fibrosis is characterized by increased proliferation of fibroblasts and excessive accumulation of extracellular matrix. Mast cell tryptase has been implicated in the development of tissue fibrosis in skin and lungs. However, the significance of mast cell tryptase in human renal diseases has not been investigated. The potential role of mast cell-derived tryptase in the development of renal fibrosis was studied using immunohistochemical techniques and cultured human renal fibroblast cell lines. Semiquantitative immunostaining analysis of samples from 70 patients with several renal diseases, including IgA glomerulonephritis (GN) (n = 30), non-IgA GN (n = 5), membranous GN (n = 5), focal segmental glomerulosclerosis (n = 4), minor glomerular abnormalities (n = 5), lupus nephritis (n = 3), and acute or chronic tubulointerstitial nephritis (n = 18), revealed that the degree of renal interstitial fibrosis was well correlated with the number of infiltrating tryptase-positive mast cells (P < 0.01). Mast cells could not be detected in damaged glomeruli in any form of renal disease. [3H]Thymidine uptake experiments demonstrated that DNA synthesis by cultured renal fibroblasts was increased with the concentration of tryptase (0.5 to 5 nM) coincubated with heparin and was suppressed by coincubation with the protease inhibitors leupeptin and benzamidine hydrochloride. Tryptase alone also increased DNA synthesis by fibroblasts but exhibited less effectiveness, compared with the combination of tryptase and heparin. Conversely, heparin alone suppressed DNA synthesis by fibroblasts. Metabolic [35S]methionine-labeling experiments with cultured renal fibroblasts indicated that tryptase increased the synthesis of fibronectin and collagen type I, in a dose-dependent manner. These findings suggest that mast cell tryptase plays a role in the proliferation and extracellular matrix protein production of renal interstitial fibroblasts and thus contributes to the development of renal interstitial fibrosis.

Renal tubulointerstitial fibrosis is the common final pathway leading to end-stage renal failure. The severity of tubulointerstitial inflammation and fibrosis has long been considered to be a crucial determinant of progressive renal injury and long-term prognoses for both human and experimental glomerulonephritis (GN) (1–3). Fibroblasts are the main effector cells in renal interstitial fibrosis. Under normal conditions, only a small proportion of these cells participate in the synthesis and deposition of extracellular matrix (ECM) proteins, mainly collagen type I (COL I), to maintain tubulointerstitial integrity (4). In pathologic conditions, fibroblasts proliferate, migrate, and synthesize growth factors as well as matrix proteins, contributing to the development of renal interstitial fibrosis (5).

Recently, several lines of evidence have suggested the participation of mast cells in fibroblast proliferation in a number of organs. Increased numbers of mast cells are observed in close proximity to proliferating fibroblasts in healing wounds and in fibrotic lesions of lungs, vessel walls, and skin in human subjects (6–9). The most abundant product of human mast cells is the serine protease tryptase. Tryptase is a tetrameric serine protease with a molecular size of 134 kD; it is composed of four monomers of 32 to 34 kD, each with one catalytic site (10). Its presence is restricted almost exclusively to mast cells, where tryptase exists within the secretary granules in a complex with heparin proteoglycan (11). Tryptase has various biologic activities, such as fibrinogenolysis, augmentation of histamine-mediated contractility of airway smooth muscle, and degradation of vasoactive intestinal peptide (12–14). Importantly, tryptase has been demonstrated to be mitogenic for fibroblasts, smooth muscle cells, and bronchial epithelial cells (15–17). Recent studies have indicated that tryptase-positive mast cells may be involved in renal interstitial injury (18–20). However, the significance of mast cell tryptase in human renal interstitial fibrosis has not been investigated. To evaluate the role of mast cell tryptase, we first examined the localization of tryptase-positive mast cells in various types of human renal diseases. In addition, we investigated the possible role of tryptase in renal fibroblast proliferation and matrix protein synthesis in vitro.

We report for the first time that mast cell tryptase may have a role in the development of renal interstitial fibrosis, because the number of infiltrating tryptase-positive mast cells was well correlated with the degree of interstitial scarring. Furthermore, we observed that mast cell tryptase is a mitogen and fibrogenic factor for human renal fibroblasts.
Materials and Methods

Patients

Immunostaining was performed on formalin-fixed, paraffin-embedded sections from 70 patients with several renal diseases, including IgA GN (n = 30), non-IgA GN (n = 5), membranous GN (n = 5), focal segmental glomerulosclerosis (n = 4), minor glomerular abnormalities (n = 5), lupus nephritis (n = 3), and acute or chronic tubulointerstitial nephritis (TIN) (n = 18).

Immunohistochemical Studies

Immunohistochemical studies were performed as described previously (21). Formalin-fixed tissue sections were deparaffinized with xylene and rehydrated with graded ethanol. Endogenous peroxidase was blocked with hydrogen peroxide, and the samples were then rinsed in phosphate-buffered saline (PBS). To yield adequate signals with the respective antibodies, the slides were trypsinized for 30 min at 37°C. For reduction of background labeling, the sections were blocked for 30 min in normal horse serum. The sections were incubated for 24 h at 4°C with mouse anti-human mast cell tryptase monoclonal antibody (Chemicon International, Temecula, CA) diluted in PBS containing 1% bovine serum albumin (1:1000). It has been demonstrated that tryptase serves as a selective marker that distinguishes mast cells from other cell types (11). After washing with PBS, the tissue was incubated with biotinylated secondary antibody, avidin-biotin-peroxidase complex (ABC Elite; Vector Laboratories, Burlingame, CA), and then 3,3′-diaminobenzidine (Dojindo, Kumamoto, Japan). Each section was counterstained with Mayer’s hematoxylin (Wako, Tokyo, Japan), dehydrated, and coverslipped. A total of 10 consecutive nonoverlapping interstitial areas (area, 0.625 mm²) were analyzed in each section of the biopsy (18). Tubulointerstitial damage was estimated on the basis of the degree of tubulointerstitial fibrosis, as primarily judged using Azan-Mallory staining. The degree of tubulointerstitial fibrosis was semiquantitatively graded by two independent observers and was expressed as a ratio of the area of fibrosis to that of the renal interstitium in biopsy sections.

Cell Culture

Human renal interstitial fibroblast cell lines were established as described previously (22). Briefly, human renal interstitial fibroblasts were obtained from the medullary part of a kidney biopsy with histologically proven GN and interstitial fibrosis. Fibroblast clones, as indicated by morphologic criteria, were subcultured in Dulbecco’s modified Eagle’s medium (Sigma Chemical Co., St. Louis, MO) with 10% fetal calf serum (FCS) (HyClone, Logan, UT). Clones of these early-passage human renal fibroblasts were immortalized by transfection with the SV40 plasmid pSVgpt (23). Stable cell lines of these SV40-immortalized fibroblasts were obtained by repeated subcloning of transfected cells (24). The renal fibroblast cell lines were comparable to the parental nontransformed interstitial fibroblasts, as indicated by similar morphologic features, unchanged patterns of expression of COL I, III, and V, fibronectin (FN), laminin, vimentin, tenascin, and intercellular adhesion molecule-1, and negative immunostaining for cytokeratin, desmin, and factor VIII (24). In addition, these fibroblasts were positive for CD90, a marker for cultured renal fibroblasts (25). The renal fibroblast cell lines (fibrosis-derived renal fibroblasts) were maintained in RPMI 1640 medium (Life Technologies/BRL, Grand Island, NY) containing 2 mM l-glutamine (Sigma), 25 mM Hepes (Sigma), 100 U/ml penicillin, 100 μg/ml streptomycin (Life Technologies/BRL), and 10% FCS. All experiments were performed after 24-h incubations in serum-free RPMI 1640 medium, to achieve fibroblast quiescence.

Proteinase Catalytic Assays

Human tryptase was purchased from Biogenesis (Poole, Dorset, UK). Tryptase activity was assayed using the peptide substrate N-tet-butoxycarbonyl-Phe-Ser-Arg-4-methyl-coumaryl-7-amide (Peptide Institute, Japan). Tryptase activity was expressed in milliunits per milliliter, where 1 U of enzyme activity was defined as the amount degrading 1 μmol of substrate per minute at 25°C (26). The specific activity of the tryptase preparations used in this study was 1.64 mU/μg.

Cell Proliferation Studies

Confluent human renal fibroblasts were detached from culture flasks with trypsin-ethylenediaminetetraacetate (Life Technologies/BRL) and were seeded into 24-well plates (Costar Corp., Cambridge, MA) at a density of 4 × 10⁴ cells/ml, in RPMI 1640 medium containing 10% FCS. At subconfluence (60%), the medium was replaced with serum-free medium for 24 h. The cells were treated with trypsin in the presence of 1 U/ml heparin (Sigma) for 24 h and were radiolabeled with 0.5 μCi/well [methyl-³²H]thymidine (DuPont/New England Nuclear, Boston, MA) for the last 12 h. The cells were collected and counted in a liquid scintillation counter (27). In experiments with protease inhibitors, tryptase was preincubated with 50 μg/ml leupeptin (a nonspecific serine protease inhibitor; Sigma) or 50 μM benzamidine hydrochloride (a relatively selective trypsin inhibitor; Sigma) for 1 h at 4°C (28). Similar experiments studying DNA synthesis by fibroblasts were performed by incubating cells with either trypsin or trypsin in the presence or absence of heparin or with heparin alone. Cell proliferation in response to incubation with trypase was also monitored by cell counting with a hemocytometer.


Growth-arrested renal fibroblasts in six-well culture plates (Costar Corp.) were incubated for 24 h in methionine-free medium containing various concentrations of trypsin with 1 U/ml heparin or heparin alone and were metabolically labeled with 100 μCi/ml [³⁵S]methionine (DuPont) for the last 18 h. For immunoprecipitation and gelatin-Sepharose absorption assays, the medium was collected and centrifuged for removal of cellular debris. Radiolabeled protein in the supernatant was quantified by counting, and a standard amount of radioactivity was used in the immunoprecipitation assays. Aliquots (150 μl) of the radiolabeled medium were diluted with 800 μl of RIPa buffer [25 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.1% sodium dodecyl sulfate (SDS), 0.5% Triton X-100, 0.5% deoxycholate]. For removal of nonspecific binding, 10 μl of normal rabbit serum and 50 μl of protein A-Sepharose (Pharmacia LKB Biotechnology, Uppsala, Sweden) were added, and tubes were incubated and centrifuged. Then, 10 μl of a rabbit antibody to human COL I (Chemicon International) was added to the supernatant, and the mixture was incubated for 16 h at 4°C. Immunocomplexes were recovered by binding to protein A-Sepharose beads (Pharmacia LKB Biotechnology). Beads were washed three times with RIPa buffer, and the immunoprecipitates were analyzed by electrophoresis on 4 to 12% SDS-polyacrylamide gels under reducing conditions, as described previously (29,30). Gelatin-Sepharose was used to isolate FN from the radiolabeled medium, as described previously (31). Briefly, 50 μl of the gelatin-Sepharose suspension was added to each sample, followed by incubation for 16 h at 4°C. The gelatin-Sepharose beads were recovered by centrifugation and washed three times with RIPa buffer. FN was released by resuspension of the beads in electrophoresis sample buffer for 3 min at 100°C, under heating conditions, and was analyzed by electrophoresis, as described above.
Northern Blot Analyses

Growth-arrested fibroblasts were treated for 24 h with either various concentrations of tryptase with 1 U/ml heparin or heparin alone. Total cellular RNA was isolated from the fibroblasts using RNAzol (Biotex Laboratories, Houston, TX). For Northern blot analysis, RNA was denatured and fractionated by electrophoresis through a 1.2% agarose gel (20 µg/lane) and was then transferred to a Hybond nylon membrane (Amersham, Buckinghamshire, UK). Nucleic acids were immobilized by ultraviolet irradiation (Stratagene, La Jolla, CA) and hybridized with $1 \times 10^6$ cpm/ml $^{32}$P-labeled cDNA probes in hybridization buffer (50% formamide, 19% Denhardt’s solution, 0.1% SDS, 5× SSC, 200 µg/ml denatured salmon sperm DNA). The cDNA probes were radiolabeled with [32P]dCTP by random oligonucleotide priming (Boehringer Mannheim Biochemicals, Indianapolis, IN). The cDNA probes used in this study included a rat α1 COL I cDNA and, for normalization of the signals obtained with the cDNA probes, a rat glyceraldehyde-3-phosphate dehydrogenase cDNA; the probes specifically bind to human α1 COL I mRNA and human glyceraldehyde-3-phosphate dehydrogenase cDNA, respectively (32). The hybridization products were observed by autoradiography. Densitometric scans were obtained from autoradiographs, for quantification of hybridization intensity.

Statistical Analyses

Statistical significance in the immunohistochemical studies (defined as $P < 0.01$) was evaluated using Pearson’s correlation coefficients. In vitro findings were presented as mean ± SD, and a value of $P < 0.05$ was used to establish statistical significance (t test). Triplicate wells were analyzed for cell proliferation studies, and each experiment was performed independently at least twice.

Results

Distribution of Tryptase-Positive Mast Cells and Renal Interstitial Fibrosis

To examine whether mast cell tryptase is involved in renal interstitial fibrosis, we first studied the localization of tryptase-positive mast cells in various renal diseases, using immunohistochemical techniques. Tryptase-positive mast cells could not be detected in patients with minor glomerular abnormalities or GN without renal interstitial fibrosis. However, mast cells were detected in the interstitium for patients with GN accompanied by interstitial fibrosis (Figure 1, A and B). Of note, many mast cells also infiltrated the fibrotic lesion of the renal

Figure 1. Immunohistochemical localization of tryptase-positive mast cells in the renal tubulointerstitium. Human mast cells were identified by immunostaining using an anti-human mast cell tryptase monoclonal antibody. These experiments were performed using kidney sections from patients with mild IgA glomerulonephritis (GN) (A), moderate IgA GN (B), acute tubulointerstitial nephritis (TIN) (C), and chronic TIN (D). Magnification, ×200.
interstitium in acute or chronic TIN (Figure 1, C and D). Mast cells were not observed in any glomeruli with or without renal diseases. Statistical analysis demonstrated that the degree of renal interstitial fibrosis was well correlated with the numbers of infiltrating mast cells in the interstitium in several renal diseases, including IgA GN, non-IgA GN, membranous GN, focal segmental glomerulosclerosis, minor glomerular abnormalities, lupus nephritis, and acute or chronic TIN \((r = 0.834, P < 0.01)\) (Figure 2). In particular, better correlations were observed for IgA GN \((r = 0.7107)\) and chronic TIN \((r = 0.64)\).

**Mitogenic Response of Human Renal Fibroblasts to Tryptase**

To investigate whether mast cell tryptase has mitogenic potential against renal fibroblasts, we examined the effect of purified tryptase on cultured human renal interstitial fibroblasts in \(^{[3]}\)Hthymidine uptake experiments. It was demonstrated that tryptase was stabilized as an enzymatically active tetramer by association with heparin and dissociated to inactive monomers without heparin (11). A dose-dependent increase in DNA synthesis by renal fibroblasts was observed after treatment with tryptase in the presence of heparin (0.5 to 5.0 nM) (Figure 3A). In addition, \(^{[3]}\)Hthymidine uptake of fibroblasts stimulated by tryptase, with or without heparin, was examined. The mitogenic activity of fibroblasts treated with tryptase without heparin was decreased, compared with that of cells coincubated with tryptase and heparin, indicating the substantial role of heparin in maintaining tryptase activity (Figure 4). Because heparin is well known to inhibit the growth of various types of cells in vivo and in vitro, we examined the effect of heparin alone on human renal fibroblasts. Heparin suppressed \(^{[3]}\)Hthymidine uptake by fibroblasts, suggesting that the mitogenic action in renal fibroblasts in this experiment is induced not by heparin but rather by tryptase with heparin (Figure 5). The proteolytic activity of tryptase was similar to that of trypsin, and this action was reported to play a role in the induction of cell signaling events by tryptase (33). Therefore, we performed an experiment to clarify whether trypsin exhibits any mitogenic effect on cultured fibroblasts. A similar dose-dependent increase in DNA synthesis by fibroblasts was observed with incubation with trypsin (10 to 50 ng/ml), but treatment of fibroblasts with higher concentrations of trypsin resulted in cell rounding and detachment from the plates (Figure 6). Finally, we examined whether the proteolytic cell signaling event induced by tryptase and trypsin at the catalytic site was directly involved in their mitogenic effects on fibroblasts. Preincuba-

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**Figure 2.** Relationship between the number of infiltrating tryptase-positive mast cells and the degree of renal interstitial fibrosis in IgA GN \((n = 30)\) (◆), non-IgA GN \((n = 5)\) (▼), minor glomerular abnormalities \((n = 5)\) (◇), membranous GN \((n = 5)\) ( ○), focal segmental glomerulosclerosis \((n = 4)\) ( ◯), lupus nephritis \((n = 5)\) ( △), acute TIN \((n = 9)\) ( ●), and chronic TIN \((n = 9)\) ( ▲). The degree of renal interstitial fibrosis was significantly correlated with the number of infiltrating mast cells \((r = 0.834, P < 0.01)\).
tion of tryptase- or trypsin-stimulated fibroblasts with the protease inhibitor leupeptin (50 μg/ml) or benzamidine hydrochloride (50 μM) resulted in a significant decrease in [3H]thymidine uptake (Figures 3 and 6), indicating that the proteolytic action of tryptase is critical for induction of the mitogenic response in fibroblasts. No significant change in mitogenesis was observed in cells incubated with either leupeptin or benzamidine hydrochloride, compared with vehicle-treated control cells (Figures 3 and 6). Similar to [3H]thymidine uptake experiments, cell-counting experiments indicated that treatment of renal fibroblasts with tryptase and heparin significantly increased cell numbers at tryptase doses of 2 and 5 nM (Figure 3B). This increased response to tryptase stimulation was suppressed by coinubcation with either leupeptin or benzamidine hydrochloride.

Effects of Tryptase on ECM Protein Synthesis and COL I mRNA Level in Human Renal Fibroblasts

To clarify whether tryptase regulates ECM protein synthesis by renal fibroblasts, we performed gelatin-absorption assays for FN and immunoprecipitation assays for COL I, using [35S]methionine-labeled renal fibroblasts. As shown in Figure 7, incubation of renal fibroblasts with tryptase and heparin for 24 h resulted in increased synthesis of FN and COL I, in a dose-dependent manner. The synthesis of FN and COL I by tryptase- and heparin-treated fibroblasts was approximately 1.9- and 2.3-fold greater, respectively, than that of control cells in response to tryptase at a dose of 2 nM. Incubation of fibroblasts with concentrations of heparin identical to those used to stabilize tryptase activity increased synthesis of COL I protein 1.6-fold, compared with control values (Figure 7). Furthermore, Northern blot analysis revealed that 24-h incubations with tryptase and heparin increased COL I mRNA levels in cultured renal fibroblasts, whereas heparin alone had no significant effect on COL I mRNA levels (Figure 8), suggesting that the increases in COL I protein levels induced by heparin may be attributable to post-translational events in heparin-treated fibroblasts (34).

Discussion

We demonstrated here for the first time that mast cell tryptase may be involved in the development of renal intersti-
tial fibrosis in various types of renal diseases, including acute or chronic TIN. In addition, we demonstrated that mast cell tryptase was not only a potential mitogen but also a stimulator of the synthesis of ECM proteins by human renal fibroblasts.

These findings from immunohistochemical assays demonstrated significant correlations between the degree of renal interstitial fibrosis and the number of infiltrating tryptase-positive mast cells, suggesting that mast cells play an important role in renal interstitial fibrotic processes regardless of the type of renal disease. These findings extended other studies that focused on the involvement of mast cells in tubulointerstitial injuries accompanied by GN, to determine the general role of mast cells in the development of tubulointerstitial fibrosis not only in GN but also in acute and chronic TIN (18–20). Of note, mast cells were mainly detected in fibrotic lesions of TIN, rather than in edematous areas. Furthermore, mast cells could not be detected in any damaged glomeruli examined. These findings strongly suggested that mast cells are unique contributors to the development of renal interstitial fibrosis. Activation of mast cells can usually be induced by multiple mechanisms, including IgE- and complement-mediated pathways. Stem cell factor (SCF) has also been identified as a differentiation, chemoattraction, and activation factor for mast cells in human subjects (35–37). Recently, Hogaboam et al. (38) reported that SCF was constitutively produced by pulmonary fibroblasts and could be induced by tumor necrosis factor-α, which is contained in mast cells. Therefore, it was suggested that activated renal interstitial fibroblasts produce SCF after injuries, to attract mast cells to injured sites.

Mast cells synthesize and secrete histamine, heparin, and various proteases and chemical mediators, such as prostaglandins, leukotrienes, and platelet-activating factors (39). Mast cell tryptase is a major protease and produces mitogenic effects on various types of cultured cells, such as smooth muscle cells and bronchial epithelial cells (16,17). Its mitogenic potential may be dependent on its proteolytic activity. The mechanism of the signaling event that mediates the fibrogenic effects of tryptase remains unclear. Like the thrombin receptor, proteinase-activated receptor-2 (PAR-2), a prototypic G protein-coupled receptor, was recently considered to be activated by proteolytic cleavage and synthetic peptides corresponding to the newly generated amino terminus after receptor cleavage (40). PAR-2 is activated by not only trypsin but also tryptase in vitro (33). Consistent with these findings, our [3H]thymidine uptake experiments demonstrated that mast cell tryptase and tryptase have mitogenic activity for human renal fibroblasts and that tryptase-mediated cellular activation requires a proteolytic event similar to thrombin-mediated signal transduction. The
development of selective PAR-2 antagonists is necessary for determination of the precise mechanisms by which tryptase induces mitogenic responses in renal fibroblasts.

It is interesting that heparin is required for induction of the full activity of tryptase in fibroblasts, because the effects of tryptase without heparin on fibroblast proliferation are less potent than those observed with heparin. Heparin is generally accepted to suppress cell proliferation in vivo and in vitro (41,42). Floege et al. (43) demonstrated that heparin suppressed mesangial cell proliferation and matrix expansion in experimental GN in rats. Castellot et al. (44) reported that heparin inhibited mitogenesis of cultured rat mesangial cells. The findings presented here also demonstrated that heparin suppressed proliferation of renal fibroblasts in vitro. Tryptase was recently demonstrated to be stabilized as an enzymatically active tetramer by association with heparin and dissociated to inactive monomers in the absence of heparin, suggesting that heparin is an essential component for the induction of tryptase action on cultured renal fibroblasts (11).

Most cells are a major source of basic fibroblast growth factor (bFGF) in chronic inflammation (45). bFGF is mitogenic for a variety of cell types, including fibroblasts, smooth muscle cells, and endothelial cells, and is known to play an important role in wound healing and fibrosis (46). Ebara and Shigematsu (19) suggested that mast cell-derived bFGF may be a key mediator in renal fibrosis. In addition, it was demonstrated that bFGF had very robust proliferative effects on renal interstitial fibroblasts, without significant effects on matrix synthesis (47). bFGF seems to be an important factor, similar to tryptase, in the development of renal fibrosis.

ECM protein deposition in the interstitium is a very important step leading to renal interstitial fibrosis. ECM deposition occurs when the balance between matrix synthesis and degradation is disturbed (48). Our findings indicated that tryptase, when coincubated with heparin, stimulated FN and COL I protein synthesis by renal fibroblasts. The increased COL I protein synthesis seemed to result from the increased COL I mRNA levels induced by tryptase. The amounts of both FN and COL I were demonstrated to increase in interstitial fibrosis of the kidney (49). Because tryptase does not alter matrix metalloproteinase expression in human dermal fibroblasts (50), this study strongly indicates that tryptase is a fibrogenic factor. Interestingly, heparin itself stimulated COL I protein synthesis but did not affect COL I mRNA expression in these experiments. This response may be attributable to the inhibitory effect of heparin on gene expression of matrix metalloproteinase-degrading ECM proteins (34).

Although it is possible that SV40-transformed human renal fibroblasts may have increased proliferative capacity, compared with primary fibroblasts, we think that our cell lines were suitable for these experiments, because our transformed fibroblasts maintained the same characteristics and produced the same ECM proteins as their primary counterparts (24).

In conclusion, we presented data indicating that mast cell tryptase may play a role in the proliferation of and ECM protein production in renal interstitial fibroblasts and may thus contribute to the development of renal interstitial fibrosis. These findings also indicated that the proteolytic activity of tryptase may be involved in the fibrogenic action of renal interstitial fibroblasts. Further studies using either administration of a neutralizing antibody against tryptase or application of a mast cell tryptase-knockout mouse strategy in experimental models of kidney fibrosis are required for more definitive determination of the role of tryptase in the development of kidney scarring.

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