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A Brief Exposure to Tryptase or Thrombin Potentiates Fibrocyte Differentiation in the Presence of Serum or Serum Amyloid P

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A key question in both wound healing and fibrosis is the trigger for the initial formation of scar tissue. To help form scar tissue, circulating monocytes enter the tissue and differentiate into fibroblast-like cells called fibrocytes, but fibrocyte differentiation is strongly inhibited by the plasma protein serum amyloid P (SAP), and healthy tissues contain very few fibrocytes. In wounds and fibrotic lesions, mast cells degranulate to release tryptase, and thrombin mediates blood clotting in early wounds. Tryptase and thrombin are upregulated in wound healing and fibrotic lesions, and inhibition of these proteases attenuates fibrosis. We report that tryptase and thrombin potentiate human fibrocyte differentiation at biologically relevant concentrations and exposure times, even in the presence of concentrations of serum and SAP that normally completely inhibit fibrocyte differentiation. Fibrocyte potentiation by thrombin and tryptase is mediated by protease-activated receptors 1 and 2, respectively. Together, these results suggest that tryptase and thrombin may be an initial trigger to override SAP inhibition of fibrocyte differentiation to initiate scar tissue formation. The Journal of Immunology, 2015, 194: 142–150.

Poorly healing chronic wounds affect >6.5 million patients in the United States each year (1). The opposite of poorly healing wounds is fibrosing diseases, where inappropriate scar tissue forms in an organ (2). Fibrosing diseases, such as pulmonary fibrosis, congestive heart failure, liver cirrhosis, and kidney fibrosis, are involved in 45% of deaths in the United States (3). Both wound healing and fibrosis involve scar tissue formation. A key component of scar tissue is the fibrocyte (4, 5). Monocytes are recruited to wounds or fibrotic lesions and differentiate into fibrocytes in response to unknown wound signals (6, 7). Fibrocytes express collagen and other extracellular matrix proteins, secrete proangiogenic factors, and activate nearby fibroblasts to proliferate and secrete collagen (4–6, 8–10).

In serum-free cultures, monocytes differentiate into fibrocytes, but the presence of as little as 0.01% serum inhibits fibrocyte differentiation (11, 12). Fibrocyte differentiation can be inhibited by the plasma protein serum amyloid P (SAP), IFN-γ, and IL-12 (12–15). The SAP concentration in plasma is ~30 μg/ml (16). The IC50 for SAP inhibition of fibrocyte differentiation is 0.2 μg/ml (12, 17), and ~1 μg/ml SAP completely inhibits fibrocyte differentiation (12). In vivo, SAP slows wound healing, whereas removing SAP from a wound promotes healing (18, 19). Conversely, SAP injections that double the serum SAP concentration inhibit fibrosis in a variety of animal models (20–23).

Normal tissues contain very few fibrocytes (10). In humans, in addition to being present in plasma, a considerable amount of SAP appears to be present in the interstitial space (24). Thus, a key question in wound healing and fibrosis is the mechanism that overrides the inhibitory effect of SAP (and other fibrocyte differentiation inhibitors) to induce fibrocyte differentiation. One of the events preceding scar tissue formation in a healing wound is the clotting cascade, in which the protease thrombin cleaves fibrinogen to fibrin. Thrombin activity is upregulated in fibrotic lesions (25) and immediately after wounding (26). Thrombin causes inflammation when added to mouse lungs, increased concentrations of thrombin within lungs exacerbate fibrosis, and inhibition of thrombin attenuates fibrosis (27–30). Thrombin cleaves a 6-aa recognition site that is found on protease-activated receptor (PAR)-1 (31, 32). This receptor is found on a variety of cell types, including monocytes (33), and it mediates the ability of thrombin to induce platelet aggregation (34).

 Mast cells are found in both internal fibrotic lesions and sites of wound healing (35–37). Mast cells degranulate in response to external stimuli (37) to release, among other things, the protease tryptase (37–39). Tryptase is upregulated in areas of increased mast cell degranulation, including wounds, and especially in fibrotic lung tissue (35, 36, 38–40). Extracellular tryptase is upregulated and associates with collagen increase in scar tissue in idiopathic pulmonary fibrosis (40). Tryptase cleaves at lysine and arginine residues, except when the subsequent amino acid is proline (41). Tryptase activates PAR-2 (37, 42). This receptor is found on a variety of cells, including monocytes (33), and it mediates the ability of tryptase to increase the proliferation of, and collagen production by, fibroblasts (37). Intratracheal administration of tryptase causes inflammation, and inhibition of tryptase attenuates this inflammation (43–46). Inhibition of PAR-2 receptors attenuates collagen deposition in a heart disease model (47).

In this study, we show that relatively brief exposures of human mononuclear cells to levels of thrombin and tryptase that would be found in a wound potentiates human fibrocyte differentiation, overriding SAP and serum inhibition; they could be a triggering mechanism for fibrocyte-mediated wound healing and fibrotic lesions.
Materials and Methods

Cell isolation and culture

Human blood was collected from volunteers who gave written consent and with specific approval from the Texas A&M University human subjects Institutional Review Board. PBMCs and monocytes were isolated as previously described (13) and cultured as previously described (48) in either serum-free medium (SFM) or protein-free medium (PFM). SFM was FibroLife Basal Medium (LifeLine Cell Technology, Walkersville, MD) supplemented with 10 mM HEPES, 1 mM nonessential amino acids, 1 mM sodium pyruvate (all from Sigma-Aldrich, St. Louis, MO), 2 mM glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin (all from Lonza, Basel, Switzerland). SFM was PFM further supplemented with 10 μg/ml recombinant human insulin (Sigma-Aldrich), 5 μg/ml recombinant human transferrin (Sigma-Aldrich), and either 550 μg/ml filter-sterilized human albumin [isolated and checked for purity as previously described (48)], fish skin gelatin (Sigma-Aldrich), or skim milk powder (EMD Millipore, Billerica, MA). Protein supplements were checked for concentration using absorbance at 280 nm. SFM made with each supplement was mixed with 500 ng/ml TPCK-treated bovine trypsin (Sigma-Aldrich) for 24 h at 37°C and assayed on 4–20% SDS gels (Bio-Rad, Hercules, CA), which were silver stained to check for purity and the presence of breakdown products. Where indicated, PFM was supplemented to 2.5% (v/v) with sterile filtered non–blood type–specific human serum, and it tested negative for hepatitis A and B and HIV I and II (Lonzar and Gemini Bio-Products, West Sacramento, CA). Monocytes were purified, tested for purity, and cultured as previously described (48, 49). Fibrocyte counts, total cell counts, and collagen staining were performed as previously described (48).

Proteases, PAR agonists, and PAR inhibitors

PAR-1 agonists SFLLRN-NH2 (American Peptide, Sunnyvale, CA) and SFLLRNPNPKYEFP (Tocris, Minneapolis, MN), PAR-2 agonists 2FLGRL-NH2 (EMD Millipore) and AC 55541 (Tocris), TPCK-treated bovine trypsin (Sigma-Aldrich) in a 1:10 molar ratio of tryptase/trypsin, human thrombin (100 U/ml, Sigma-Aldrich), and acetyl-55541 (Tocris, Minneapolis, MN, 1 mM), 10,000 BAEE U/mg, and human thrombin (100 U/ml, Sigma-Aldrich) were resuspended following the manufacturer’s instructions. After a 1-h incubation on ice in PFM, PBMCs were collected by centrifugation at 300 × g for 10 min, resuspended in ice-cold PBS, and washed at 300 × g for 10 min, twice. PBMCs were then plated and allowed to differentiate under the indicated conditions, as described previously (48).

SAP and IFN-γ

Human SAP was purified, as previously described (12), with the following modifications. Human serum from blood donors was mixed 1:1 with PBS. This was mixed with gentle rolling in a 10:1 ratio with SP Sepharose beads (GE Healthcare, Piscataway, NJ) in 20 mM Tris, 140 mM NaCl, and 2 mM CaCl2 (pH 8) and eluted as previously described (12). SAP purity was checked by silver stain on an SDS-PAGE gel, and concentration was assessed by absorbance at 280 nm. Proteases at 40 ng/ml were coincubated with purified SAP, and the reactions were analyzed by PAGE with 4–20% SDS gels and silver stained to assess cleavage of SAP. Recombinant human IFN-γ (PeproTech, Rocky Hill, NJ) was resuspended according to the manufacturer’s instructions. Then SAP and IFN-γ were added to SFM at the indicated concentrations.

Transwell migration

Transwell migration of monocytes was performed as previously described (53), with the following modifications: the filter size was 8 μm, SFM was used as medium, cells were allowed to pass through the filter for 12 h, and adherent cells that passed through the filter were imaged with an IN Cell 2000 microscope (GE Healthcare) and counted by CellProfiler (54).

Statistics

Statistics were performed using Prism (GraphPad, San Diego, CA). Differences were assessed by two-tailed t tests or Mann–Whitney tests. Significance was defined as p < 0.05.
fibrocytes was accompanied by an increase in the number of collagen-positive cells (Fig. 2). The number of adherent cells following fixing and staining was not significantly affected by tryptase or thrombin (Supplemental Fig. 1), suggesting that tryptase and thrombin specifically increase the number of differentiated fibrocytes, rather than increasing general cell viability or adhesion.

The plasma thrombin concentration in a clotting wound is ~37 nM (26). The molecular mass of thrombin is ~36 kDa, so this thrombin concentration is ~1.3 × 10³ ng/mL. Far from a wound, the levels of active thrombin should be negligible, so assuming that a gradient of thrombin forms in the interstitial space, with the highest concentration in the clotting blood, at some point in the tissue near the wound the thrombin concentration would be on the order of the 3–50 ng/mL that potentiates fibrocyte differentiation in vitro (Fig. 1). Tryptase requires stabilization by heparin to be enzymatically active (50). Thus, tryptase (~500 kDa) signaling must be measured by its activity. In wound fluid, tryptase activity is ~0.30 mU/mg wound fluid protein, where a unit is the cleavage of 1 μmol/min of Z-gly-pro-arg-pNA (61). In the assay conditions used in a previous study (61), tryptase activity was ~1.75 × 10⁸ mU/g/tryptase, and 35 nM tryptase cleaved 30 pmol/s of 0.2 mM Z-gly-pro-arg-pNA (62). Wound fluid contains ~31 mg/mL protein (63), and combining these indicates that wound fluid contains ~63 ng/mg tryptase, within the range of tryptase concentrations that promote fibrocyte differentiation in vitro (Fig. 1). Together, these results suggest that physiological concentrations of thrombin and tryptase can potentiate fibrocyte differentiation.

Fibrocytes differentiate from monocytes (4, 6, 11). Cells in the PBMC population include T cells, B cells, monocytes, and NK cells (12). To determine whether trypsin, tryptase, or thrombin acts directly on monocytes to potentiate fibrocyte differentiation, we purified monocytes from PBMCs by negative selection, as previously described (48). Monocytes were 16 ± 9% (mean ± SEM, n = 3) of the PBMCs (92 ± 5% in the purified fraction). Trypsin, tryptase, and thrombin all potentiated the differentiation of fibrocytes from purified monocyte populations by a factor of ~2 (Fig. 3), similar to the ~2-fold increase in fibrocytes that these proteases caused in PBMC populations (Fig. 1). Together, these results suggest that tryptase, tryptase, and thrombin directly affect monocytes to potentiate fibrocyte differentiation.

Tryptase and thrombin potentiate fibrocyte differentiation in serum-containing media

We previously observed that trypsin potentiates fibrocyte differentiation in medium supplemented with human serum (48). Serum FIGURE 2. Tryptase and thrombin increase collagen secretion by PBMCs. (A) Representative flow plots of no-protease control PBMCs stained with control rabbit IgG (black) and for collagen (green) and protease-treated PBMCs stained for collagen (red). PBMCs were exposed to 12.5 ng/ml tryptase (B) or thrombin (C) for 5 d, resuspended for collagen, and assayed by flow cytometry. Values are mean ± SEM (n = 5). *p < 0.05, t test.

Results

Tryptase and thrombin potentiate fibrocyte differentiation

We previously observed that trypsin, but not chymotrypsin, pepsin, or endoprotease GluC, potentiates fibrocyte differentiation (48). Fibrocytes are involved in wound healing, and trypsin is used topically to speed wound healing (55–60). The extracellular levels of both tryptase and thrombin increase during the formation of scar tissue (26, 35). To determine whether tryptase and thrombin also potentiate fibrocyte differentiation, we examined the effect of these proteases on fibrocyte differentiation in culture. Our previous assays had been done in media with a supplement called ITS-3; because of difficulties in obtaining this, we substituted human albumin for ITS-3 (48). Human PBMCs were incubated with trypsin, tryptase, or thrombin for 5 d in SFM containing human albumin. The cells were then stained and scored for fibrocyte formation. In the absence of added proteases, we observed 388–1120 fibrocytes/10⁵ PBMCs from the different donors, similar to what we observed previously (11, 13, 14, 48). Because of this variability, fibrocyte numbers were normalized to protease-free controls. In media with albumin as the supplement, 0.2–445 ng/mL trypsin potentiated fibrocyte differentiation (Fig. 1A, 1D), encompassing the 20–200 ng/mL trypsin concentrations that we previously observed to increase fibrocyte differentiation in media with ITS-3 (48). Tryptase concentrations between 4 and 56 ng/mL and thrombin concentrations between 3 and 50 ng/mL also increased the number of fibrocytes significantly (Fig. 1B–D). Tryptase concentrations above 100 ng/mL and thrombin concentrations above 400 ng/mL decreased the number of fibrocytes (Fig. 1B, 1C). These effects were observed for all donors tested. In addition to having a unique morphology, fibrocytes express collagen, and the protease-induced increase in the number of visible
is present in a wound after blood clots (64). To determine whether tryptase and thrombin can potentiate fibrocyte differentiation in an environment containing serum, we incubated PBMCs in serum-containing medium for 5 d with tryptase and thrombin. As previously observed (12), serum inhibited fibrocyte differentiation (Fig. 4). In the presence of serum, 14–446 ng/ml tryptase and 28–224 ng/ml thrombin potentiated fibrocyte differentiation (Fig. 4), indicating that the levels of these proteases that would be observed in a wound can override the inhibitory effect of serum.

**Tryptase potentiates fibrocyte differentiation in the absence of albumin**

The observation that three different proteases, in the presence of albumin, potentiate fibrocyte differentiation suggests that either the proteases potentiate fibrocyte differentiation or that a proteolytic fragment of albumin potentiates fibrocyte differentiation. To determine whether fibrocyte potentiation is dependent on the protein composition of the defined medium, we coincubated PBMCs with trypsin and fish gelatin or trypsin and skim milk powder. Tryptsin caused fibrocyte potentiation when mixed with fish gelatin or skim milk powder (Supplemental Fig. 1). There is only a 15% sequence similarity between human albumin and fish gelatin, and the largest identical sequence is 3 aa. This suggests that, after proteolytic digestion of the two proteins, there is no common peptide produced that could activate fibrocyte differentiation. The observation that trypsin potentiates fibrocyte differentiation in the presence of fish gelatin and the absence of albumin suggests that albumin is not necessary for the trypsin effect; thus, trypsin may directly potentiate fibrocyte differentiation.

**FIGURE 4.** Tryptase and thrombin potentiate fibrocyte differentiation in the presence of human serum. PBMCs were cultured in medium containing serum in the presence of the indicated concentrations of tryptase or thrombin for 5 d, and fibrocytes were counted as in Fig. 1. Serum-containing media completely inhibited fibrocyte differentiation in the no-protease control. Values are mean ± SEM (n = 4). *p < 0.05, **p < 0.01, ***p < 0.001 versus no-protease control, t test.

**FIGURE 5.** PAR-1 and PAR-2 affect fibrocyte differentiation. PBMCs were incubated with inhibitors of PAR-2 (A–C) or inhibitors of PAR-1 (D–F) before mixing with trypsin (A and D), tryptase (B and E), or thrombin (C and F) at the indicated concentrations. PAR-1 agonists (G) or PAR-2 agonists (H) were added to PBMCs at the indicated concentrations. After 5 d, fibrocytes were counted as in Fig. 1. Values are mean ± SEM (n = 6). In (G) and (H), arrows indicate the published EC$_{50}$ concentrations. *p < 0.05, **p < 0.01, ***p < 0.001 versus agonist-free or protease-free control (t test) for ENMD-1068, SCH 79797, SFLLRN-NH$_2$, and 2f-LIGRL-NH$_2$. *p < 0.05 for FSLLRY-NH$_2$, vorapaxar, SFLLRNDKYPF, and AC 55541 (each n = 3).
Trypsin, tryptase, and thrombin signal through PARs

Because trypsin, rather than a proteolytic fragment of albumin, appears to potentiate fibrocyte differentiation, we examined the possibility that trypsin activates a cell surface receptor. Proteases can act as extracellular signals by cleaving the extracellular domains of PARs (65). PAR-1 and PAR-2 are expressed on human monocytes (33). To determine whether trypsin, tryptase, or thrombin potentiates fibrocyte differentiation through PAR-1 or PAR-2, we examined the effect of these proteases on PBMC differentiation after exposure to PAR-1 and PAR-2 inhibitors. ENMD-1068 and FSLLRY-NH2 are peptides that selectively block PAR-2 activation but do not interfere with the activities of PAR-1, PAR-3, or PAR-4 (37, 47). ENMD-1068 (at 26 nM) and FSLLRY-NH2 (at 12 nM) inhibit PAR-2 function, as measured by proliferation and collagen production in fibroblasts (37, 47). Twenty-six nM ENMD-1068 and FSLLRY-NH2 decreased trypsin and tryptase potentiation of fibrocyte differentiation (Fig. 5A, 5B), but thrombin potentiation of fibrocyte differentiation was not significantly affected (Fig. 5C).

SCH79797 and vorapaxar are inhibitors of PAR-1 that do not interfere with PAR-2 signaling (51, 52). SCH79797 (at 70 nM) and vorapaxar (at 47 nM) inhibited PAR-1 function in platelet-aggregation assays (51, 52). SCH79797 and vorapaxar did not significantly affect trypsin’s or tryptase’s fibrocyte potentiation (Fig. 5D, 5E), but they blocked the ability of thrombin to potentiate fibrocyte differentiation (Fig. 5F). Conversely, PAR-1 and PAR-2 agonists potentiates fibrocyte differentiation at concentrations similar to those previously observed to be effective (66–69) when added to PBMCs (Fig. 5G, 5H) or to purified monocytes (Fig. 3). These data suggest that tryptase and trypsin signal through PAR-2, and thrombin signals through PAR-1, to potentiate fibrocyte differentiation.

Tryptase and thrombin compete with SAP to potentiate fibrocyte differentiation

SAP inhibits the differentiation of monocytes into fibrocytes, whereas trypsin, tryptase, and thrombin potentiate this differentiation. Tryptase and thrombin are present in wounds along with SAP (26, 35). To determine how these signals might compete with each other, we coincubated SAP with proteases. As previously observed (12), 10 μg/ml SAP completely inhibited fibrocyte differentiation (Fig. 6A–F). In the presence of 10 μg/ml SAP, trypsin showed no significant potentiation of fibrocytes from PBMCs (Fig. 6A), but it did potentiate fibrocyte differentiation from monocytes (Fig. 6F). Levels of tryptase and thrombin that would be observed in a wound, as well as PAR-1 and PAR-2 agonists, competed with 10 μg/ml SAP to potentiate fibrocyte differentiation from PBMCs (Fig. 6B–E) and purified monocytes (Fig. 6F). This potentiation was not caused by protease digestion of SAP, because only trypsin caused any measurable cleavage of SAP (Fig. 6G).

To determine whether trypsin, tryptase, or thrombin changes the IC50 of SAP’s inhibition of fibrocyte differentiation, we coincubated each protease with a series of SAP concentrations (Fig. 7, FIGURE 6. Tryptase and thrombin compete with SAP to potentiate fibrocyte differentiation, but they do not obviously digest SAP. PBMCs mixed with SAP at 10 μg/ml were incubated with the indicated concentrations of trypsin (A), tryptase (B), thrombin (C), PAR-1 agonist SFLLRN-NH2 (D), or PAR-2 agonist 2f-LIGRL-NH2 (E) for 5 d. Fibrocytes were then counted as in Fig. 1. Values are mean ± SEM (n = 4), *p < 0.05, **p < 0.01, ***p < 0.001 versus SAP with no protease or no agonist, t test. (F) Monocytes in SFM were coincubated with 10 μg/ml SAP in the absence (control) or presence of 12.5 ng/ml protease or 10 μM PAR-1 or PAR-2 agonist. After 5 d, fibrocytes were counted as in Fig. 3. Values are mean ± SEM (n = 3), *p < 0.05, **p < 0.01, ***p < 0.001 versus SAP control, t test. (G) Purified SAP was incubated with proteases at 40 ng/ml for 24 h at 37˚C, run on a 4–20% SDS-PAGE gel, and stained with silver. Molecular masses (kDa) are indicated on the left.
Supplemental Fig. 1E). Trypsin, tryptase, thrombin, PAR-1 agonist, and PAR-2 agonist significantly potentiated fibrocyte differentiation in the presence of SAP at some of the indicated SAP concentrations (Fig. 7). SAP inhibited fibrocyte differentiation with an IC₅₀ of 0.16 ± 0.05 μg/ml. SAP’s IC₅₀ was 0.23 ± 0.13 μg/ml with trypsin, 0.39 ± 0.13 μg/ml with tryptase, 1.03 ± 0.46 μg/ml with thrombin, 0.44 ± 0.31 μg/ml with PAR-1 agonist, and 1.59 ± 0.77 μg/ml with PAR-2 agonist. The effects of tryptase, thrombin, and PAR-2 agonist on the IC₅₀ were significant (p < 0.05, Mann–Whitney U test). Together, these results indicate that tryptase and thrombin can compete with SAP to potentiate fibrocyte differentiation.

Trypsin, tryptase, thrombin, and PAR-1 and PAR-2 agonist compete with IFN-γ to potentiate fibrocyte differentiation

Like SAP, IFN-γ is present in wounds and scar tissue (70, 71), and it inhibits the differentiation of monocytes into fibrocytes (15). To determine how these signals might compete with each other, we coincubated proteases with IFN-γ. As previously observed, 10 ng/ml IFN-γ inhibited fibrocyte differentiation (15). Each protease and receptor agonist potentiated fibrocyte differentiation in the presence of 10 ng/ml IFN-γ (Supplemental Fig. 2).

To determine whether proteases or PAR agonists act as monocyte chemoattractants or chemorepellents, we placed PBMCs in the upper cup of a Boyden chamber and added each protease or agonist above, below, or on both sides of the filter. Each protease reduced PBMC migration through the filter, suggesting that these proteases may have a chemostatic effect on PBMCs, regardless of whether the protease was added above or below the filter (Supplemental Fig. 2). Each agonist acted as a chemoattractant whether added above or below the filter (Supplemental Fig. 2).

A brief exposure to trypsin, tryptase, or thrombin potentiates fibrocyte differentiation

Tryptase and thrombin are proteolytically active over short time frames in wounds and in scar tissue (26, 50). To determine whether a brief exposure to these proteases is sufficient to potentiate fibrocyte differentiation, we allowed PBMCs to adhere to plates and exposed them to tryptase, trypsin, or thrombin for 4 h (Fig. 8), 12 h (Supplemental Fig. 3), or 24 h (Supplemental Fig. 4). Subsequently, the medium was completely removed and fresh, protease-free medium was added to the PBMCs for the rest of the 5-d assay. These conditions approximate the bursts of time that...
proteases would be active in a fibrotic lesion or a healing wound environment.

At each time point, tryptase, trypsin, and thrombin were able to potentiate fibrocyte differentiation over a broader range than the range established by the 5 d exposure to proteases (Fig. 1). Similarly, proteases at each time point were able to significantly potentiate fibrocyte differentiation in the presence of 2.5 μg/ml SAP or 2.5% (v/v) human serum. These data suggest that even a brief exposure to tryptase, trypsin, or thrombin over biologically relevant concentrations is sufficient to induce fibrocyte differentiation.

**Discussion**

In this study, we show that tryptase and thrombin potentiate fibrocyte differentiation and collagen production, as well as that this potentiation even occurs in the presence of levels of serum or SAP that completely inhibit fibrocyte differentiation. Tryptase and thrombin potentiation appears to act directly on monocytes and is mediated by PAR-2 and PAR-1, respectively. Tryptase and thrombin potentiate fibrocyte differentiation at biologically relevant concentrations and exposure times. Not all proteases are profibrotic, because pepsin and chymotrypsin do not activate PAR-1 or PAR-2 (72–74), and do not potentiate fibrocyte differentiation (48). These results suggest a triggering mechanism for fibrocyte-mediated wound healing and fibrotic lesions, by which thrombin from clotting blood or tryptase from a sufficient amount of mast cell degranulation overrides SAP inhibition and initiates fibrocyte differentiation.

Tryptase, thrombin, PAR-1 signaling, and PAR-2 signaling have been implicated in the development of fibrosis through their effects on fibroblasts (25, 31, 32, 37, 40, 75–78). Both PAR-1 and PAR-2 have been implicated in liver fibrosis in mice (79, 80), and both PAR-1-knockout mice and PAR-2–knockout mice are less susceptible to induced heart disease and inflammation (81–83). Intratracheal administration of trypsin, tryptase, and thrombin causes inflammation, and inhibition of tryptase and thrombin attenuates this inflammation (27, 30, 43, 45). Inhibitors of tryptase (84–86) and thrombin (87), as well as antagonists of both PAR-1 (88) and PAR-2 (89), are patented for the treatment of fibrosis.

Both tryptase (90) and thrombin (91) inhibitors are in clinical trials for the treatment of fibrosis. However, although PAR-1 and PAR-2 antagonists have been suggested as therapeutics (92, 93), neither is in clinical trials. Thus, PAR-1 and PAR-2 mediate both fibroblast proliferation and fibrocyte differentiation, two major components of scar tissue (49, 94–96). Our work strongly supports and expands the idea that tryptase, thrombin, PAR-1 signaling, and PAR-2 signaling potentiate wound healing and fibrosis.

Systemic mastocytosis involves the degranulation of mast cells throughout the body (97) and is associated with serious local, as well as moderate systemic, fibrosis (98, 99). Mast cell degranulation causes the release of tryptase. Serum tryptase is ~2 ng/ml in healthy patients, whereas it is 20–100 ng/ml in mastocytosis patients (100, 101). Our observation that 4–56 ng/ml tryptase potentiates fibrocyte differentiation suggests that the fibrosis seen in mastocytosis patients may be due to the released tryptase inducing fibrocyte differentiation.

That tryptase and thrombin compete with SAP suggests that the PAR-1 or PAR-2 pathway is capable of potentiating fibrocyte differentiation in the presence of SAP. Tryptase potentiates fibrocyte differentiation in the presence of SAP after a brief exposure (Fig. 8, Supplemental Figs. 3 and 4) but not over the course of a 5-d differentiation (Figs. 6A, 7A). Tryptase and thrombin potentiated fibrocyte differentiation under all time course, SAP, and serum conditions. Tryptase and trypsin appear to signal through the same receptor (37). It is unclear whether our results imply that different PAR-2 isoforms exist or that PAR-2 can differentiate between trypsin and tryptase signaling.

A protein additive was necessary for fibrocyte potentiation. Although albumin mixed with protease was the most effective media treatment for potentiating fibrocyte differentiation, both fish gelatin and milk powder also increased fibrocyte differentiation when mixed with protease. This suggests that the fibrocyte potentiation caused by proteases relies on a suitable protein additive, not solely albumin. Albumin is increased in fibrotic lesions (25) and healing wounds (102), and it is decreased in chronic, nonhealing wounds (103–105), implying that its presence may mediate the protease signaling that, by activating fibrocyte differentiation, may initiate key aspects of wound healing and fibrosis.

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**Disclosures**

The authors have no financial conflicts of interest.

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PROTEASES TRIGGER FIBROCYTE DIFFERENTIATION


