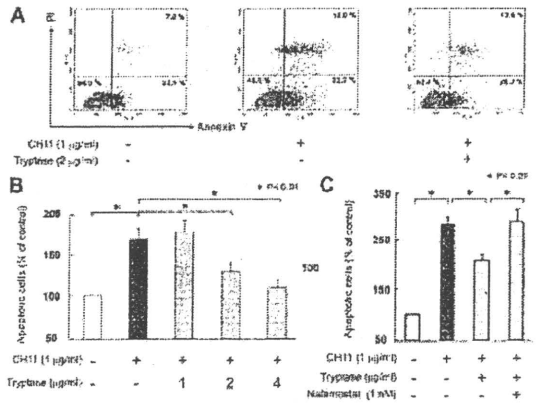

Figure 4. Inhibition of anti-Fas antibody–mediated early and late apoptosis of rheumatoid arthritis synovial fibroblasts (RASFs) by tryptase. **A,** RASFs were left untreated (**left**) or treated with anti-Fas antibody in the absence (**middle**) or presence (**right**) of tryptase. Twelve hours after treatment, RASFs were stained with annexin V/propidium iodide (PI) and analyzed for apoptosis by flow cytometry. **B,** Apoptosis of RASFs was determined in cultures of cells left untreated (controls) or treated with anti-Fas antibody in the absence or presence of various doses of tryptase, with results normalized to untreated control values (set at 100%). Bars show the mean and SD results from 5 different RA synovial tissue samples. **C,** E11 fibroblasts were left untreated or treated with anti-Fas antibody in the absence or presence of tryptase with or without nafamostat mesylate. Twelve hours after treatment, RASFs were stained with annexin V and analyzed for the percentage of apoptotic (annexin V–positive) cells by flow cytometry. Bars show the mean and SD results from triplicate determinations in each condition, with results normalized to untreated control values (set at 100%).

4A). Both early and late apoptosis were inhibited by tryptase in a concentration-dependent manner (Figure 4B). Of note, apoptosis of primary dermal fibroblasts could not be induced under the same conditions (results not shown), suggesting that this phenomenon might be specific to RASFs. Taken together, these results suggest that tryptase inhibits Fas-induced apoptosis in RASFs.

We next tested whether tryptase inhibits Fas-mediated apoptosis of fibroblasts via the activation of PAR-2. Since PAR-2 is activated by proteolytic cleavage of the receptor, we tested whether the addition of the protease inhibitor nafamostat mesylate would reverse the antiapoptotic effects of tryptase. As expected, treatment of fibroblasts with anti-Fas antibody resulted in cell death, and this was attenuated by the addition of tryptase (Figure 4C). However, the protective effect of tryptase was lost when the cells were cotreated with tryptase and nafamostat mesylate (Figure 4C), suggesting that the proteolytic function of tryptase and subsequent cleavage of PAR-2 is responsible for the antiapoptotic effects of tryptase against Fas-mediated apoptosis of fibroblasts.

Involvement of Rho activation in the antiapoptotic effect of tryptase on RASFs. Activation of Rho, which is a low molecular weight G protein, is related to cell survival (16,17). We have previously reported that activation of Rho through the ligation of PAR-1 by thrombin promotes proliferation of RASFs (18). Since Rho also mediates downstream signaling of PAR-2 (19), the activation of Rho in tryptase-stimulated RASFs was evaluated. The activation of Rho was examined with the use of a pull-down assay for the detection of GTP-bound Rho (active form of GTPases) followed by Western blot analysis of the Rho protein. An increase in GTP-bound Rho was observed in RASFs after treatment with tryptase (Figure 5), suggesting that PAR-2 stimulation induces the activation of Rho.

We then tested whether the activation of Rho is involved in the protection of RASFs against CH11-induced apoptosis. After the addition of CH11 to the RASF cultures, an increase in annexin V^{high} cells was observed, and this was again inhibited by tryptase (Figure 6A). The protective effect of tryptase was abrogated by the addition of a Rho kinase–specific inhibitor, Y27632, in a dose-dependent manner (Figures 6A and B). Similar results were obtained in RASFs from 5 separate RA patients (results not shown). Taken together, these data suggest that tryptase inhibits Fas-induced apoptosis of RASFs through a mechanism that involves Rho kinase.

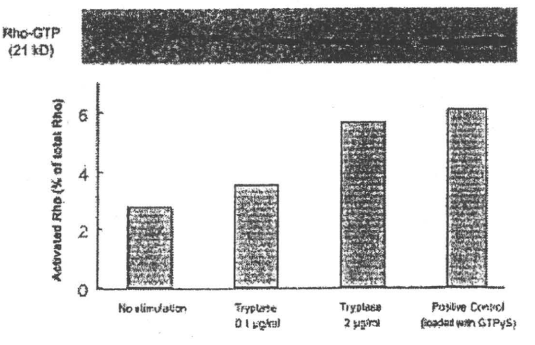

Figure 5. Activation of Rho kinase by tryptase in rheumatoid arthritis synovial fibroblasts (RASFs). RASFs were treated with tryptase (0.1 or 2 μg/ml) for 1 minute (or treated with GTPγS as a positive control). **Top,** After cell lysis, GTP-bound Rho was assessed using a pull-down assay involving the Rhotekin-Rho binding domain. **Bottom,** The ratio of activated Rho to total Rho protein was determined. Equal loading of Rho in the pull-down assay was confirmed by Western blotting. Representative results from 1 of 3 RA patients are shown.

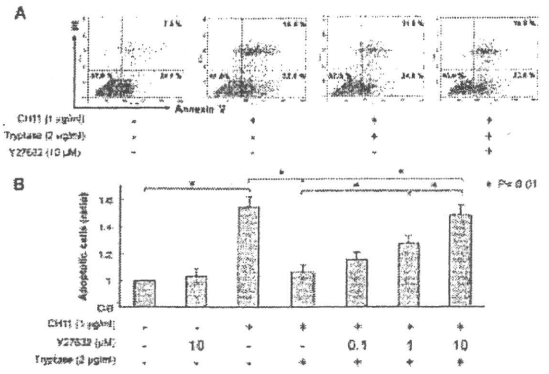

Figure 6. Rho kinase–dependent tryptase-mediated inhibition of apoptosis. **A,** Rheumatoid arthritis synovial fibroblasts (RASFs) were left untreated **(left)** or treated with anti-Fas antibody in the absence or presence of tryptase **(middle panels)** or treated with anti-Fas antibody in the presence of tryptase and the Rho kinase inhibitor Y27632 **(right)**. Twelve hours after treatment, RASFs were stained with annexin V/propidium iodide (PI) and analyzed for apoptosis by flow cytometry. **B,** The ratio of apoptotic cells to total cells was determined in experiments using RASFs from 5 different patients, with results normalized to untreated control values. Bars show the mean and SD.

DISCUSSION

Although numerous mast cells are present in RA synovial tissue, their involvement in the pathogenesis of RA remains unclear. In this report, we shed light on one potential mechanism by which mast cells may contribute to RA. Since tryptase is a protease that is specifically produced by mast cells and is considered one means by which mast cells can convey information to surrounding cells, we hypothesized that tryptase and its receptor, PAR-2, may play a role in RA pathogenesis. Through our studies, we have demonstrated that tryptase-expressing mast cells lie in close proximity to RASFs in the synovial tissue of RA patients. Furthermore, RASFs express the receptor for mast cell tryptase (PAR-2) and are protected from Fas-mediated apoptosis by tryptase in a Rho kinase–dependent manner. Such a mechanism could play an important role in the marked proliferation of RASFs and hyperplasia seen in RA synovium, leading to disease progression.

One reason that we focused on the effect of mast cell mediators on SF apoptosis was to yield insight into the apparently paradoxical finding that RASFs proliferate vigorously in vivo despite the high expression of Fas (2,3). Moreover, RASFs are readily susceptible to anti-Fas–mediated apoptosis in vitro (20). These findings suggest that a mechanism that prevents Fas-mediated cell death exists in RASFs, and that this excessive proliferation may contribute to disease pathogenesis. Indeed, we were able to demonstrate that RASFs isolated from 5 independent patients with RA exhibited apoptosis when incubated with anti-Fas antibody. However, Fas-mediated apoptosis was inhibited by a mast cell–specific protease, tryptase. Thus, we propose that the accumulation of mast cells in RA synovium creates an environment that is rich in tryptase and allows RASFs to counteract Fas-mediated killing. In fact, the interplay between RASFs and mast cells may be important for the maintenance of chronic inflammation in the RA synovium.

The PARs represent a unique family of receptors that are activated by proteolytic cleavage (21). The ligand for these receptors is encoded in the N-terminal region of the receptor itself but is unable to bind until the N-terminus is cleaved at specific sites by serine proteases, such as thrombin and tryptase (22). The proteolytic cleavage of PARs creates a new N-terminus that can now bind to this G protein–coupled receptor, and subsequently activates the small G protein Rho. In our present study, several lines of evidence suggest that tryptase protects RASFs against Fas-mediated apoptosis through PAR-2. First, PAR-2 was found to be expressed both in vivo and ex vivo on RASFs. Second, the inhibition of the protease function of tryptase by the protease inhibitor nafamostat mesylate reversed the protective effect conferred by tryptase. Third, Rho was activated upon tryptase treatment of RASFs. Finally, the anti-apoptotic effect of tryptase was abrogated by the addition of the Rho kinase inhibitor Y27632. We have previously reported that thrombin-mediated PAR-1 activation also allows RASF survival and proliferation through a similar mechanism (18), by inhibiting apoptosis through the activation of Rho (16,17). Taken together, these results suggest the possibility that a series of protease-mediated signals is important in the pathogenesis of RA.

Obviously, RASFs are not the only cells that contribute to joint destruction and inflammation, since the pannus is a complex inflammatory granulation tissue (23) consisting of RASFs, vascular tissue, and an inflammatory cell infiltrate. However, it is noteworthy that PAR-2 is also expressed on other cells in the pannus, including vascular endothelial cells and inflammatory cells (24,25). Thus, it is tempting to speculate that mast cells also affect other cell types in the synovium of RA patients, through a tryptase/PAR-2–dependent mechanism.

Our present findings are consistent with those from previous studies in animal models, in which it has

been demonstrated that tryptase is involved in the pathogenesis of murine joint inflammation. For example, mice lacking monocyte chemotactic protein 6 (analogous to human tryptase) show resistance to antibody-mediated arthritis (26), suggesting that mouse mast cell proteases play an important role in joint inflammation. Furthermore, the injection of tryptase directly into the joints of mice results in inflammation and swelling. However, this inflammation is not observed when tryptase is injected into PAR-2–deficient mice, suggesting that tryptase can cause joint inflammation through the activation of PAR-2 (27).

Our study demonstrates a potential mechanism by which mast cells contribute to RA pathogenesis, through their communication with RASFs. Numerous mast cells reside in close proximity to PAR-2–expressing RASFs in the synovium of RA patients. We believe that the interaction of mast cell–associated tryptase and PAR-2 on RASFs inhibits the apoptosis of RASFs, causing hyperplasia of RA synovial tissue. This notion is consistent with the observation that, similar to neoplastic cells, RASFs multiply, and this occurs even though RASFs express high levels of Fas (2,3). Although further studies are required to test whether such interactions indeed occur in vivo, we propose that therapy aimed at inhibiting the mast cell/tryptase/PAR-2/Rho pathway may be a new treatment target for patients with RA.

ACKNOWLEDGMENT

The authors thank Ms T. Adachi for providing excellent technical assistance.

AUTHOR CONTRIBUTIONS

All authors were involved in drafting the article or revising it critically for important intellectual content, and all authors approved the final version to be published. Dr. Tanaka had full access to all of the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

Study conception and design. Sawamukai, Tanaka.
Acquisition of data. Sawamukai, Yukawa.
Analysis and interpretation of data. Sawamukai, Saito, Nakayamada, Kambayashi.

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