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Autocrine Inhibition of Differentiation in HL-60 Cells by Secreted Proteinase 3

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Various molecules have been reported to be involved in the induction of HL-60 cell differentiation by all-*trans* retinoic acid (ATRA). Notably, the transfection of an antisense oligonucleotide targeting proteinase 3 (PRTN3, formerly known as myeloblastin) into HL-60 cells induces differentiation, though the underlying mechanism remains unclear. Here we found that the levels of secreted PRTN3 were significantly reduced when differentiation was initiated by ATRA treatment, suggesting that secreted PRTN3 inhibits HL-60 cell differentiation. The addition of anti-PRTN3 antibody to the culture medium of HL-60 cells promoted their differentiation. Conversely, when ATRA-treated HL-60 cells were cultured in conditioned media prepared from HEK293 cells transfected with the preproPRTN3 plasmid, differentiation was significantly suppressed. Moreover, conditioned media from HEK293 cells transfected with an inactive PRTN3 mutant (S203G) also suppressed HL-60 cell differentiation. These results suggest that PRTN3 secreted from HL-60 cells has an autocrine inhibitory effect on differentiation, independent of its enzymatic activity.

Key words all-*trans* retinoic acid, differentiation, HL-60 cells, proteinase 3

INTRODUCTION

Acute myeloid leukemia is characterized by abnormalities in myeloblasts, leading to uncontrolled proliferation of leukemia cells.[1,2] Among the various types of acute myeloid leukemia, acute promyelocytic leukemia (APL) is notable for the presence of the PML-RARα fusion gene, which is caused by a chromosomal translocation. This fusion gene halts differentiation and promotes neoplastic proliferation at the promyelocyte stage. Differentiation therapy using all-*trans* retinoic acid (ATRA) is predicated on the idea that differentiation-inducing agents can prompt cancer cells arrested in an immature or poorly differentiated state to resume maturation.[2,3]

Arsenic trioxide (ATO) is used to treat APL,[4,5] and several studies have reported the efficacy of combining ATO with ATRA for this purpose.[6] To investigate how ATO contributes to differentiation, we examined its effect on HL-60 cells.[7] The results indicated that the combination of ATRA and ATO induced greater differentiation than ATRA alone, as evidenced by the expression of several differentiation-related factors in HL-60 cells. In particular, co-treatment with ATO reduced the expression of proteinase 3 (PRTN3) to 0.18-fold compared to ATRA alone. PRTN3 is a neutral serine proteinase in the azurophil granules of neutrophils and monocytes.[7] It is known to mature and activate major inflammatory cytokines, such as membrane-bound TNF, IL-1β, and IL-18, through secretion outside the cell. It has been implicated in inflammatory diseases such as type II diabetes, cardiovascular disease, rheumatoid arthritis, and atherosclerosis.[8] In addition, PRTN3 is involved in the differentiation of leukemia cells. Bories *et al*.[9] reported that the introduction of antisense oligonucleotides targeting PRTN3 into HL-60 cells induces their differentiation. Although the mechanism remains uncertain, Liu *et al*.[10] recently reported that PRTN3 suppresses STAT3-dependent differentiation by promoting STAT3 degradation via ubiquitination.

In this study, we focused on the secretion of PRTN3 and conducted experiments to examine whether extracellular PRTN3 suppresses the differentiation of HL-60 cells. The results showed that differentiation was induced by adding a PRTN3 antibody to the culture medium of HL-60 cells, and that differentiation was suppressed when HL-60 cells were cultured in conditioned media prepared from HEK293 cells transfected with the preproPRTN3 gene. These findings indicate that secreted PRTN3 contributes to the arrest of differentiation in HL-60 cells.

MATERIALS AND METHODS

Materials All-*trans* retinoic acid was purchased from Sigma-Aldrich (St. Louis, MO). The PE-anti-human CD11b antibody was purchased from BioLegend (San Diego, CA). The PRTN3 antibody (#MAB6134), mouse IgG_{2A} isotype control (#MAB003), and human PRTN3 ELISA Kit were purchased from R&D Systems (Minneapolis, MN). The c-Myc (#017-21871) and GAPDH (#016-25523) antibodies were pur-

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chased from Wako Pure Chemical (Osaka, Japan). All other reagents and chemicals used were of the highest grade available.

Cell Culture HL-60 cells were obtained from ATCC (Manassas, VA) and cultured at 37°C in a humidified atmosphere of 5% CO_2 using RPMI-1640 (Wako Pure Chemical) containing 10% fetal calf serum, penicillin (100 U/ml), and streptomycin (100 μg/ml). HL-60 cells were authenticated by the JCRB Cell Bank at the National Institute of Biomedical Innovation (Osaka, Japan). HEK293 cells were obtained from the RIKEN BRC Cell Engineering Division (Ibaraki, Japan) and cultured at 37°C in a humidified atmosphere of 5% CO_2 using Dulbecco's modified Eagle's medium (Wako Pure Chemical) containing 10% fetal calf serum, penicillin (100 U/ml), and streptomycin (100 μg/ml).

Flow Cytometry The levels of cell differentiation antigen CD11b were determined using flow cytometry (Guava easyCyte 6HT/2L; Millipore, Billerica, MA). Cells (7.5×10^5 cells/500 μL D-PBS) were incubated with 4 μL of PE-anti-human CD11b antibody for 30 min at 4°C, washed, and then subjected to flow cytometry. To quantify the percentage of cell populations retaining CD11b expression on the cell surface, the cell populations were divided into quadrants, and the percentages of cells shifting from the lower-left to the lower-right quadrant were calculated.

Human PreproPRTN3 Cloning and Mutagenesis Human preproPRTN3 cDNA was amplified from total RNA extracted from HL-60 cells by RT-PCR. The total RNA was isolated, and 3 μg of total RNA was reverse-transcribed to cDNA and amplified with PrimeSTAR™ Max DNA Polymerase (Takara, Shiga, Japan) using the primers. The sequences for the sense and antisense primers were 5'-GATTCATGGCTCACCGGCCCCCCAGCCC-3' and 5'-GGTACCGGGCGGCCCTTGGCCTCCACACG-3', respectively. The sequenced cDNAs of preproPRTN3 were subcloned into p3×FLAG-Myc-CMV-26 (Sigma Aldrich) for overexpression of the preproPRTN3 protein with a FLAG tag at the *N*-terminus and a c-Myc tag at the *C*-terminus in HEK293 cells. The mutation from Ser at position 203 to Gly (S203G) was introduced using specific primers and a QuickChange site-directed mutagenesis kit (Agilent Technologies, Santa Clara, CA). The resultant construct was verified by DNA sequencing. The primer sequences for mutagenesis were 5'-ATCTGCTTCGGAGACGGAGGTGGCCCCCTGATC-3' and 5'-GATCAGGGGGCCACCTCCGTCTCCGAAGCAGAT -3'.

Transient Transfection HEK293 cells were seeded at 2.4×10^6 cells per well in 10 cm dishes and transfected with 8 μg of each plasmid per dish, along with 80 μL Polyfect transfection reagent (QIAGEN, Valencia, CA). PRTN3 protein expression was confirmed by Western blotting using anti-c-Myc and PRTN3 antibodies.

Western Blotting Proteins were separated by SDS-PAGE. Gels were transferred to an immunoblot PVDF membrane and placed in a blocking solution consisting of TBST (10 mM Tris (pH 8.0), 150 mM NaCl, and 0.05% Tween 20) with 5% skim milk for 1 h. The blotted membranes were incubated with the appropriate antibody, washed with TBST, and incubated with an HRP-conjugated secondary antibody. Bound IgG was visualized using Western blotting detection reagents (Thermo Scientific, Waltham, MA) according to the manufacturer's protocol.

PRTN3 Levels in HL-60 and HEK293 Cell Supernatants After exposure of HL-60 cells to ATRA and transfection of HEK293 cells with the preproPRTN3 plasmid, the media were collected. PRTN3 protein levels were measured using the PRTN3 ELISA Kit according to the manufacturer's protocol.

Statistical Analysis Data were obtained from three or four separate experiments. Values are presented as means ± SEM. Statistical significance was assessed using one-way ANOVA followed by Dunnett's test (Fig. 1C) and Tukey–Kramer post-hoc testing (Fig. 3D, 4B, and 4C) and Student's t-tests (Fig. 2 and Fig. 3B) Differences between groups were considered statistically significant at $p < 0.05$.

RESULTS

To measure differentiation after treatment with ATRA (0.1 and 1 μM) for 48 h, HL-60 cells were stained with CD11b and analyzed by flow cytometry (Fig. 1A). The ATRA treatment increased the percentage of CD11b-positive cells. With differentiation confirmed, PRTN3 protein levels were measured during the induction of differentiation in HL-60 cells treated with ATRA (Figs. 1B and 1C). Western blot analysis revealed that PRTN3 levels were decreased in a dose- and time-dependent manner (Fig. 1B), and the amount of PRTN3 in the medium was also significantly decreased 48 h after ATRA treatment (Fig. 1C). These results indicated that extracellularly secreted PRTN3 in the culture medium decreased during the differentiation process in HL-60 cells treated with ATRA.

The possibility that secreted PRTN3 suppresses differentiation of HL-60 cells was assessed. The differentiation of HL-60 cells was measured by adding an anti-PRTN3 antibody to the culture medium (Fig. 2). The results showed that the anti-PRTN3 antibody significantly induced differentiation of HL-60 cells compared to the mouse IgG_{2A} isotype control. These findings suggest that extracellularly secreted PRTN3 suppresses HL-60 cell differentiation.

We further investigated whether extracellularly secreted PRTN3 suppresses HL-60 cell differentiation using conditioned media. HEK293 cells were transfected with the preproPRTN3 plasmid, and PRTN3 protein was detected in both the cells and the culture media. As shown in Fig. 3A, Western blot analysis using antibodies against c-Myc (the C-terminal tag) and PRTN3 confirmed the presence of PRTN3 protein in cells (Fig. 3A, lane 2) and in the culture medium (Fig. 3A, lane 4) following transfection. In addition, ELISA confirmed that PRTN3 protein was significantly induced in the culture medium of cells transfected with the preproPRTN3 plasmid (Fig. 3B). The culture medium was collected after transfection into HEK293 cells as conditioned medium, and HL-60 cells treated with ATRA for 24 h were cultured in this conditioned medium (Fig. 3C). When we measured the differentiation of HL-60 cells after 24 h of culture in conditioned medium, we found significant suppression of HL-60 cell differentiation in the conditioned medium from preproPRTN3-transfected cells compared to that from vector-transfected cells (Fig. 3D). The results shown in Fig. 3D and Fig. 2 clearly demonstrate that extracellularly secreted PRTN3 suppresses HL-60 cell differentiation.

Since it has been reported that the 203rd serine is involved in the enzymatic activity of PRTN3,[11] we next used WT and S203G mutants to investigate whether PRTN3 activi-

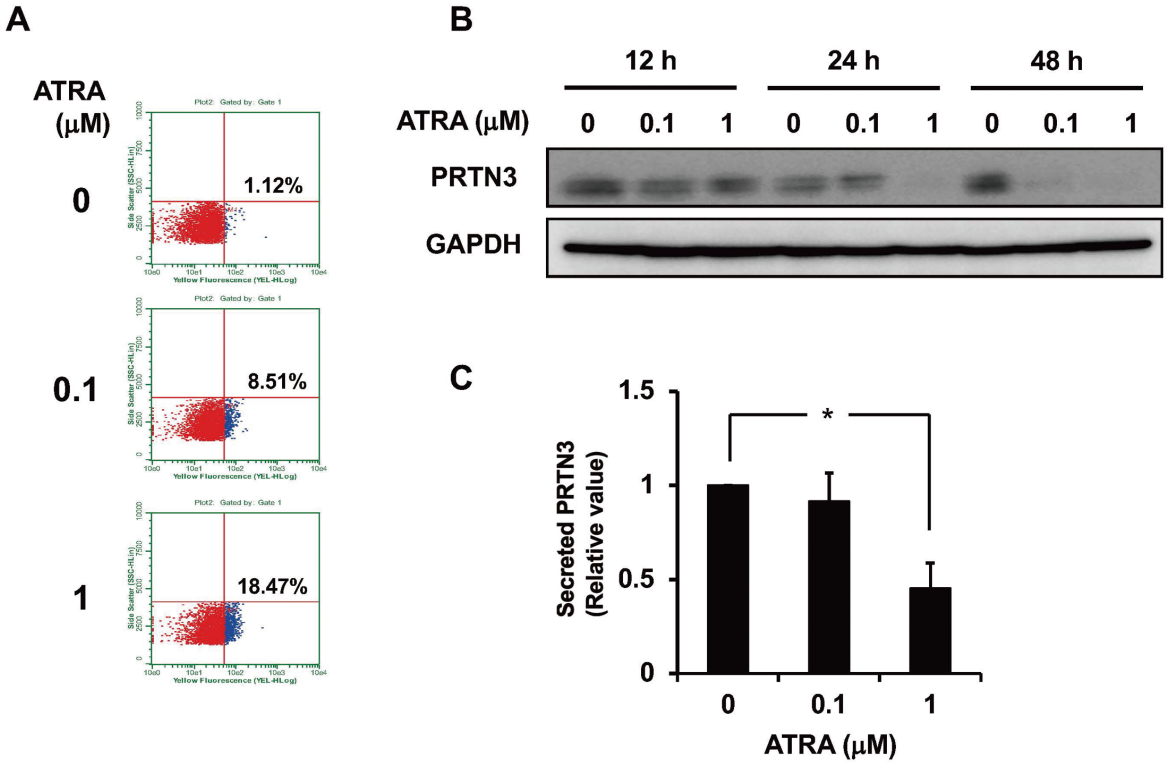

Fig. 1. Levels of PRTN3 during Differentiation of HL-60 Cells with ATRA

A: HL-60 cells (7.5×10^5 cells) treated with the indicated concentrations of ATRA for 48 h were incubated with PE-anti-human CD11b antibody. Surface expression of CD11b on treated HL-60 cells was analyzed by flow cytometry. The percentage of cell populations retaining CD11b expression was quantified. Data are presented as a percentage of CD11b-positive cells when the populations were divided into quadrants. The numbers in the figure show representative results for the percentage of CD11b-positive cells. **B:** HL-60 cells (7.5×10^5 cells) treated with the indicated concentrations of ATRA for 12, 24, and 48 h. Western blot analyses were performed for PRTN3. GAPDH was detected as a loading control. **C:** HL-60 cells (1×10^5 cells) were treated with the indicated concentrations of ATRA for 48 h, and the medium was collected. PRTN3 levels were measured with a human PRTN3 ELISA Kit according to the manufacturer's protocol. Data are presented as relative values to control cells. *p<0.05.

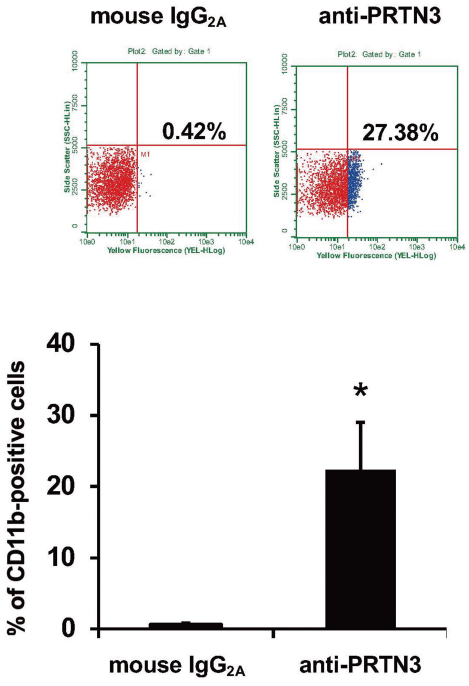

Fig. 2. Differentiation of HL-60 Cells by Adding PRTN3 Antibody to the Culture Medium

HL-60 cells (7.5×10^5 cells) were treated with 10 μg of anti-PRTN3 antibody for 48 h. The mouse IgG_{2A} isotype control (10 μg) served as a negative control. Treated cells were incubated with PE-anti-human CD11b antibody. Surface expression of CD11b on treated HL-60 cells was analyzed by flow cytometry, and the percentage of cells retaining CD11b expression was quantified. Data are presented as the percentage of CD11b-positive cells, with populations divided into quadrants. The figure shows representative results for the percentage of CD11b-positive cells. *p<0.05.

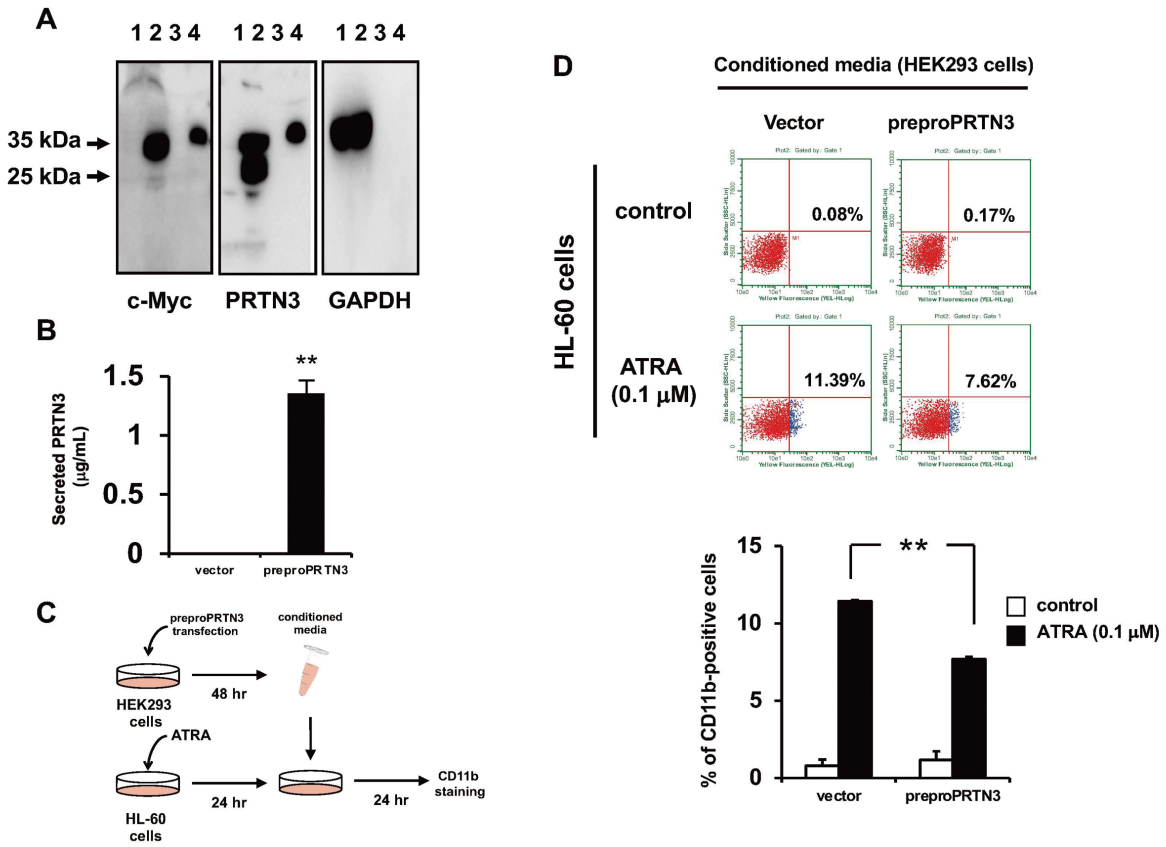

Fig. 3. Differentiation of HL-60 Cells after the Addition of Conditioned Medium from HEK293 Cells Transfected with PreproPRTN3

HEK293 cells were transfected with the preproPRTN3 plasmid for 48 h. **A:** The cells and culture medium were collected, and Western blots were performed using anti-c-Myc and PRTN3 antibodies. Lanes 1 and 2 show intracellular proteins after transfection with the vector and preproPRTN3, respectively, while lanes 3 and 4 show culture medium after transfection with the vector and preproPRTN3. **B:** Culture media obtained after transfection with vector and preproPRTN3 were used to measure PRTN3 levels by ELISA. $^{**}p<0.01$. **C:** Schematic of the differentiation experiment for HL-60 cells using conditioned media containing PRTN3. **D:** HEK293 cells were transfected with the vector and the preproPRTN3 plasmid for 48 h, and the culture media were collected. HL-60 cells treated with ATRA (24 h) were divided into two groups, and conditioned medium obtained from transfected HEK293 cells was added to each group, followed by further incubation for 24 h. Treated cells were incubated with PE-anti-human CD11b antibody, and surface expression of CD11b on treated HL-60 cells was analyzed by flow cytometry. The percentage of cells retaining CD11b expression was quantified. Data are presented as the percentage of CD11b-positive cells after the cell populations were divided into quadrants. The numbers in the figure show representative results for the percentage of CD11b-positive cells. $^{**}p<0.01$.

ty is required for the inhibition of HL-60 cell differentiation. After transfecting WT and S203G into HEK293 cells, we confirmed that the levels of intracellular PRTN3 were nearly equal (Fig. 4A) and that the levels of secreted PRTN3 protein were also comparable (Fig. 4B). HEK293 cells were transfected with WT or S203G, and the conditioned media were collected. When HL-60 cells were cultured in conditioned media containing WT or S203G (as shown in Fig. 3D), the inhibitory effect on HL-60 cell differentiation observed with conditioned media from WT-transfected cells was also detected in conditioned media from S203G-transfected cells (Fig. 4C). These results suggest that the enzymatic activity of PRTN3 is not required for the inhibitory effect of conditioned media containing PRTN3 on HL-60 cell differentiation.

DISCUSSION

APL is a disease in which differentiation is suppressed at the promyelocyte stage, resulting in uncontrolled proliferation of neoplastic cells.[12] Administration of ATRA, a first-line treatment, initiates the arrest of differentiation. In a study exploring the usefulness of combination therapy with ATRA and ATO[13]—which is also used for APL—we found that PRTN3 expression levels were significantly reduced by the combination of ATO and ATRA compared with ATRA alone.[7] Although PRTN3 previously showed an inhibitory effect on differentiation, based on the observation that transfection with antisense-PRTN3 stimulated differentiation in HL-60 cells, the mechanism by which this occurs remains unclear.[9] In this study, we investigated whether extracellularly secreted PRTN3 might have an inhibitory effect on differentiation.

Lutz PG *et al.*[14] reported that adding ATRA to HL-60 cells suppressed PRTN3 expression. When measuring the promoter activity of PRTN3, they found that the PU.1, C/EBP, and Myb binding domains were active, indicating that Myb is a master regulator. Since ATRA also reduced the Myb expression level, the reduction in PRTN3 expression might be mediated by decreased Myb levels[14]. Although different leukemia cells were used, a similar mechanism is likely at work in HL-60 cells.

Addition of anti-PRTN3 antibody to the culture medium of HL-60 cells promoted their differentiation (Fig. 2). Furthermore, replacing the culture medium of HL-60 cells, which had been differentiated by adding ATRA for 24 h, with culture medium containing PRTN3 inhibited differentiation (Fig. 3D). These findings suggest that secreted PRTN3 is involved in the inhibition of differentiation. Since differentiation was also inhibited with an inactive PRTN3 mutant

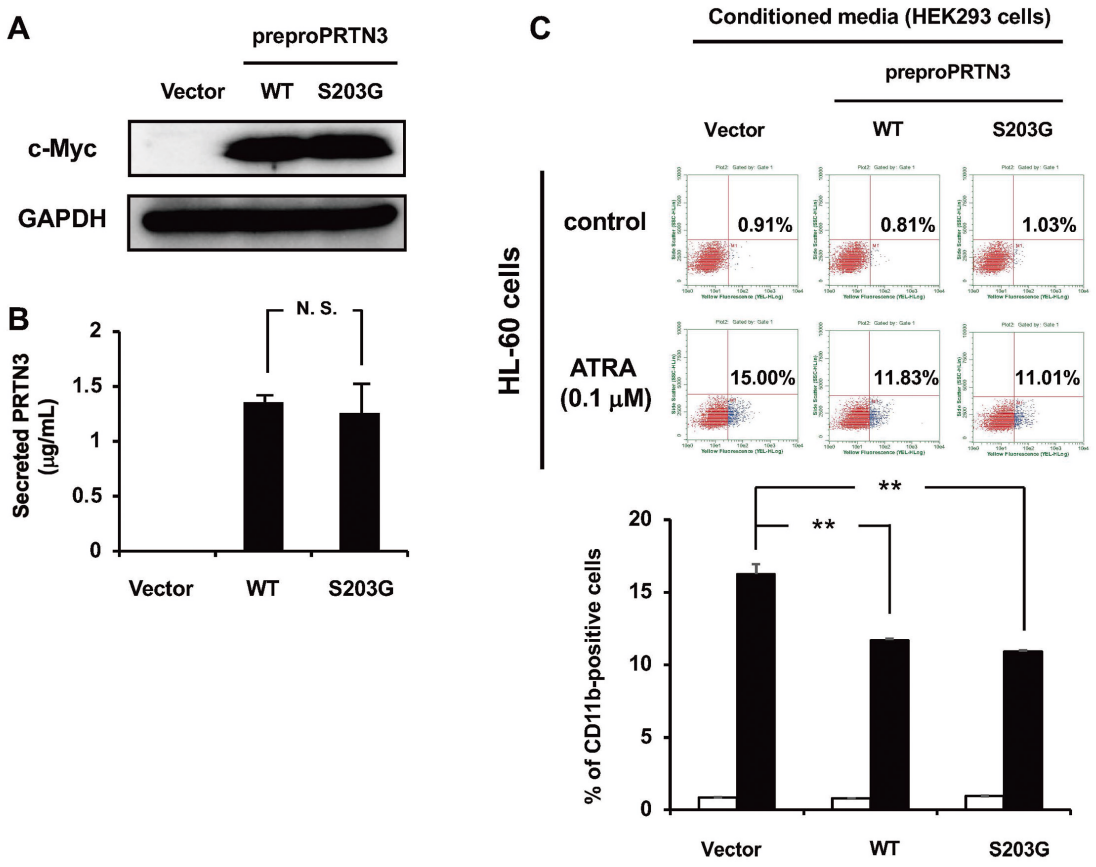

Fig. 4. Comparison of Differentiation of HL-60 Cells Treated with Conditioned Media of PRTN3 and Its Mutants

HEK293 cells were transfected with the preproPRTN3 (WT) and S203G mutant plasmids for 48 h. **A:** The culture media were collected, and Western blots were performed using an anti-c-Myc antibody. **B:** Culture media obtained after transfection with the vector, preproPRTN3 (WT), and S203G mutant were used to measure PRTN3 levels by ELISA. N.S. indicates not significant. **C:** HEK293 cells were transfected with the vector, preproPRTN3 (WT), and S203G mutant plasmids for 48 h, after which the culture media were collected. HL-60 cells treated with ATRA for 24 h were divided into three groups, and conditioned medium obtained by transfecting HEK293 cells was added to each group, followed by further incubation for 24 h. Treated cells were incubated with PE-anti-human CD11b antibody. Surface expression of CD11b on treated HL-60 cells was analyzed by flow cytometry. The percentage of cellular populations that retained CD11b surface expression was quantified. Data are presented as a percentage of CD11b-positive cells after the cell populations were divided into quadrants. The numbers in the figure show representative results for the percentage of CD11b-positive cells. $^{**}p<0.01$.

(Fig. 4C), it appears that the PRTN3 protein itself inhibits differentiation. Recently, Liu H *et al.*[10] reported that in the arrest of differentiation in leukemia cells, intracellular PRTN3 binds to the differentiation initiation factor STAT3, leading to STAT3 degradation. Combined with our present findings, this fact suggests not only that translated PRTN3 exists in HL-60 cells, but that secreted PRTN3 enters HL-60 cells via endocytosis and binds to STAT3. Further investigation is needed to determine whether secreted PRTN3 is internalized by endocytosis and induces STAT3 degradation.

Regarding the three bands detected in lane 2 stained with anti-PRTN3 antibody (Fig. 2A), we believe that these bands are products derived from the transfection of preproPRTN3 gene, since these bands were not detected in lane 1. In addition, compared to lane 2 stained with anti-c-Myc antibody, no bands around 25 kDa or smaller were detected, so it is thought that the cleavage occurred at a position toward the *N*-terminus from the c-Myc attached to the *C*-terminus. As a candidate for the cleavage site, the propeptide is 249-256 in the Uniprot database (https://www.uniprot.org/uniprotkb/P24158/entry), so this area is likely to be cleaved. However, if cleavage at this area is considered to be important for the secretion of PRTN3, it is thought that nothing was detected in lane 4 stained with anti-c-Myc antibody, so it is expected that the propeptide cleavage site at the *C*-terminus is not functional. Regarding why bands around 25 kDa and smaller bands are not detected in lane 4 when stained with anti-PRTN3 antibody (Fig. 2A), considering that these small bands are truncated toward the *N*-terminus from the c-Myc attached to the *C*-terminus, we expect that the cleaved area contains an important for secretion. According to the above database, asparagine 129 and 174 are predicted to be glycosylated, so it is possible that these amino acids are missing due to cleavage.

The results in Fig. 4C revealed that PRTN3 activity is not involved in the inhibition of HL-60 cell differentiation by secreted PRTN3. PRTN3 has been reported to contribute to the shedding of cytokines such as TNF-α and IL-1β.[8] TNF-α is known to regulate the HL-60 cell cycle,[15] and our results also showed that IL-1β levels were higher with ATO treatment than with ATRA alone,[7] suggesting that IL-1β may be involved in HL-60 cell differentiation. However, considering that PRTN3 enzymatic activity is not involved in the inhibition of differentiation, activation of these cytokines by other proteases may contribute to HL-60 cell differentiation.

In this study, we found that secreted PRTN3 is involved in the inhibition of differentiation of HL-60 cells. It was also revealed that this inhibitory mechanism is independent of PRTN3's enzymatic activity. Based on these findings, we

believe that developing nucleic acid drugs to suppress PRTN3 expression—rather than using PRTN3 inhibitors—would be beneficial in the treatment of leukemia associated with PRTN3.

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Conflict of interest The authors declare no conflict of interest.

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