Soluble form of the ST2 gene product exhibits growth promoting activity in NIH-3T3 cells

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1. Introduction

The ST2 gene was originally identified as a gene induced during the initiation of cell proliferation of quiescent BALB/c-3T3 cells, and thus was named ST2, short for serum TIMulation-2 [1,2]. Since a subsequent investigation revealed that the ST2 gene also encodes the type I transmembrane protein, ST2L (the Long form of ST2), which has a sequence very similar to the interleukin 1 receptor, the focus of the research has shifted to the immunological aspects [3]. IL-33 was found after a long search for ligand(s) to ST2L [4], promoting further research into the immune function(s) of ST2/ST2L in relation to IL-33 signaling [5].

On the other hand, using an ELISA system for the human ST2 protein [6], we found that the ST2 level was increased in serum as well as other body fluids of patients suffering from various disorders, such as bronchial asthma, systemic lupus erythematosus, acute eosinophilic pneumonia, idiopathic pulmonary fibrosis, malignant pleural effusions, and subarachnoid hemorrhage [7–12]. Most surprising was the association between ST2 and myocardial infarction, which has led to the ST2 level being used as a biomarker to predict heart failure [13,14]. However, the spectrum of diseases that are accompanied by high ST2 levels in the serum and body fluids is too broad to be explained merely by immunological function(s) of the ST2 gene products. In fact, we found that ST2 suppresses colony formation of T98G glioblastoma cell line in soft agar [15], and that the ST2 is directly internalized into dendritic cells [16], and both these findings suggest that ST2 has a unique function other than the sequestration of IL-33 [5].

In the present study, therefore, we investigated the relation between cell growth regulation and the original findings for the ST2 gene. In the course of a previous cell proliferation assay using NIH-3T3 cells, IL-33 was found to have dual functions with respect to cell growth – namely, a suppressive effect on the growth initiation of quiescent cells, and a growth-promoting effect on cycling cells [17]. However, it remains to be determined whether ST2 and ST2L play any roles in cell growth regulation. Here we report that ST2 has a growth-promoting effect in the NIH-3T3 fibroblastic cell line.

2. Materials and methods

2.1. Cell culture

The NIH-3T3 cell line, which can be introduced into G0 phase, was used [17,18]. Each experiment was started by thawing a new
vial of cells. After 2 days, the cells were split in a ratio of ca. 1:5 (6 × 10^5 cells per culture dish) in a 10-cm culture dish containing 10 mL of DMEM (D6429; Sigma-Aldrich Co., St. Louis, MO) supplemented with 5% (v/v) heat-inactivated FBS (HyClone; HyClone Lab. Inc., Logan, UT), 100 U/mL of penicillin (Gibco Life Technologies Co., Tokyo, Japan), and 100 μg/mL of streptomycin (Gibco), and cultured at 36.5 °C in a 5% CO_2 atmosphere for 5 days. Cell counting was performed using a Scepter™ handheld automated cell counter (Millipore Corp., Billerica, MA) throughout the experiments.

2.2. Cell proliferation assay

The cells cultured for 5 days as described above were washed twice with PBS, and re-suspended in an appropriate volume of DMEM plus 5% FBS to 1 × 10^5 cells/mL. After careful stirring, 0.5 mL of the suspension was added to each well of the 24-well cell culture plates (Costar 24 Well Clear TC-Treated Multiple Well Plates #3526; Corning Inc., Corning, NY), resulting in precisely 5 × 10^3 cells/well (a ca. 1:20 splitting ratio). Blank control wells, each containing 0.5 mL of the medium without cells, were also prepared in each plate.

Cell proliferation was measured using a Premix WST-1 cell proliferation assay system (MK400; Takara Bio, Otsu, Japan). At the appropriate time, each well was supplemented with 50 μL of WST-1 solution, and then the plate was carefully shaken 10 times and kept at 36.5 °C in a 5% CO_2 atmosphere. After 2 h, the plate was again shaken carefully, and then the solution in each well was mixed well by pipetting. One hundred-microliter samples were transferred to a 96-well ELISA plate (SUMILON; Sumitomo Bakelite Co., Ltd., Tokyo, Japan), and processed for measurement of the absorbance at 450 nm/620 nm using an ImmunoMini NJ-2300 (InterMed Japan Inc., Osaka, Japan). When the absorbance was expected to be over 2, the assay was terminated at 1 h and the absorbance was doubled to ensure linearity of the assay.

Three measurements were carried out for each well, and three independent wells were analyzed for a particular time point. Every experiment was repeated at least 3 times beginning with thawing of the frozen cells. The excellent correspondence between the WST-1 assay and cell counting method has already been confirmed in our previous report [17].

2.3. RNA extraction

Total RNA was extracted using TRIzol Reagent (Life Technologies, Carlsbad, CA). The cells were cultured and treated with a 1:20 splitting ratio in the same manner as in the cell proliferation assay, with the exception that 10-cm-diameter cell culture dishes were used instead of 24-well plates. Since the concentration of RNA in the extract was expected to be low due to the sparseness of the culture, RNA grade glycogen (#R0551; Thermo Scientific) was used as a carrier in the precipitation process using isopropanol. The final concentration of RNA dissolved in H_2O was measured with a NanoDrop 2000c spectrophotometer (Thermo Scientific).

2.4. Reverse transcription and PCR (RT-PCR)

One microgram of the total RNA was denatured at 70 °C for 10 min and immediately chilled on ice. The first-strand DNA was synthesized at 37 °C for 1 h in a total volume of 20 μL using oligo (dT)₁₂₋₁₈ primer (Sigma-Aldrich) and M-MLV Reverse Transcriptase (Life Technologies) as described previously [19]. PCR amplification was carried out with synthesized cDNA derived from 0.05 μg of total RNA in a final volume of 20 μL using an Advantage 2 PCR Kit (Clontech, Mountain View, CA) [20]. The standard PCR conditions were 95 °C for 10 min, followed by 30 cycles of 94 °C for 1 min, 55 °C for 1 min, and 72 °C for 2 min, and a final treatment at 72 °C for 10 min. The nucleotide sequences of the forward primers were as follows: for ST2 and ST2L (derived from a common region of mouse ST2 and ST2L in the exon 6) [2, 3, 21], 5′-CAATCTCTCCA-TACACACAC-3′; and for GAPDH, 5′-CCATCCATCTTCAGGAGG-3′.

Five microliters of PCR products were developed by electrophoresis on 1.2% agarose gels, and the gels were stained with ethidium bromide.

2.5. Quantitative PCR analysis

Quantitative PCR by using KAPA SYBR Fast qPCR kit (KAPA Biosystems, Wilmington, MA, USA) was performed in a LightCycler 96 (Roche Diagnostics, Indianapolis, IN, USA) with PCR cycles set at 95 °C for 10 s, 55 °C for 10 s, and 72 °C for 10 s. The nucleotide sequences of the forward primers used for the quantitative PCR were as follows: for ST2 (in exon 8), 5′-TCAAGCATTGTTGAGGGAC-3′; for ST2L (in exon 9), 5′-AGGTGCTAATATCTTCCTC-3′; and for GAPDH, 5′-CCT-GCTTACCACCTCTTGTG-3′.

All primer sets are on different exons, thereby avoiding the effect of contamination of genomic DNA in RNA preparations, if any. The expected lengths of the PCR products were 353 bp for ST2, 458 bp for ST2L, and 576 bp for GAPDH, respectively.

Five microliters of PCR products were developed by electrophoresis on 1.2% agarose gels, and the gels were stained with ethidium bromide.
2.6. Retrovirus production and infection

Human embryonic kidney 293T cells (HEK293T) were transfected with helper retrovirus plasmids including pE-Eco and pGP (TAKARA, Japan) together with MSCV-ires-PuroR (murine stem cell virus (MSCV)-internal ribosome entry site (IRES)-puromycin N-acetyltransferase vectors) encoding ST2-FLAG or ST2L-FLAG proteins tagged with FLAG on their C-termini. The viruses were harvested from culture supernatants and stored on ice. Exponentially growing cells (1 \times 10^5 cells per 60-mm-diameter cell culture dish) were infected with virus-containing supernatant in complete medium containing 1.0 \ \mu g/mL Polybrene (Sigma-Aldrich, St. Louis, MO). The infection efficiencies were confirmed by puromycin selection. The resulting transfected cells were designated as NIH-ST2 and NIH-ST2L, respectively. The cells transfected with an empty virus as a control were designated NIH-MSCV.

2.7. Immunoblotting

The infected cells were lysed with RIPA buffer (10 mM sodium phosphate, pH 7.2, 150 mM NaCl, 2 mM EDTA, 1% NP-40, 1% sodium deoxycholate, 0.2 U/ml aprotinin, and phosphatase inhibitors). To detect secreted ST2, the culture supernatant was also collected. These samples were electrophoretically separated by SDS-PAGE and transferred to polyvinylidene difluoride membranes (Merck Millipore). Proteins were detected with M2 monoclonal antibody against Flag tag (M2; Sigma-Aldrich).

2.8. Purification of recombinant ST2 protein

To purify the recombinant ST2 protein, the culture supernatant of NIH-ST2 cells infected with the retrovirus harboring ST2-FLAG was collected. Similarly, the culture supernatant of control NIH-MSCV cells was also harvested to prepare a control fraction. These culture supernatants were mixed with 50 \ \mu L of M2-agarose (Sigma-Aldrich) at 4 °C for 90 min. The precipitated complexes were washed with PBS five times, and then the fraction including ST2-FLAG was eluted by the addition of PBS including 400 \ \mu g/mL of FLAG peptide (Sigma-Aldrich). The purity and approximate protein amount of purified ST2-FLAG were evaluated by Coomassie Brilliant Blue (R-250) staining with bovine serum albumin as a standard protein for the calibration after SDS-PAGE.

2.9. RNA interference

Annealed oligonucleotides coding shRNA for both murine ST2 and ST2L were inserted into a pSUPER-retro-puro retroviral plasmid (Oligoengine, Seattle, WA). The retroviruses were prepared by transfection into HEK293T cells, and utilized for the infection into NIH-3T3 cells by the same method as used for MSCV retroviral plasmids. The efficiency of knockdown was evaluated by RT-PCR and immunoblot analysis with an M2 antibody. The sequences of oligonucleotides used for constructing the shRNA retroviral vectors for KD (knockdown) 2 cells were 5’-GATCCCaggaatatcctggtgacatTTCAGAGAgtcaccacagttacctTTTTA-3’ and 5’-AGCTTAAAAaaggatatcctggtgacatTCTCTTGAgtcaccacagttacctGGG-3’ (underlined sequences correspond to

Fig. 2. Expression of ST2 and ST2L in the derivative cells of NIH-3T3 cells. (A) Quantitative PCR analysis was performed as in Fig. 1B. (B) FLAG-tagged ST2 and ST2L proteins in conditioned media (CM) and whole cell lysate (WCL) from the infected NIH-3T3 cells were detected by immunoblotting with M2 antibody.
the sequence of murine ST2, from 876 to 894 in ORF) [2]. The target sequences for KD1 and KD3 cells were `acacaatggaagtggaaat` (677–695) and `agaggaaggtcgaaatgaa` (819–837), respectively. As control, an shRNA construct against firefly luciferase was used in KD0 cells. To express shRNA-resistant ST2 and ST2L in KD2-ST2/ST2L-knockdown cells, five nucleotides in the shRNA target sequence were mutated to avoid RNAi targeting (Fig. 4B). The mutations did not alter the amino acid sequences of ST2 and ST2L.

2.10. Cell cycle analysis

For analysis of synchronized cells, NIH-3T3 cells were arrested at G0 phase by subjecting them to contact inhibition during culture to confluence. The synchronized cells were released into the cell cycle by splitting the cells onto new dishes. At the indicated periods, cells were fixed and suspended with the lysis buffer including propidium iodide and RNase A for flow cytometry. Cell cycle data were collected using LSFortessa (Becton Dickinson, Franklin Lakes, NJ) and analyzed with FlowJo software version 10.0.8 (Tree Star Inc., Ashland, OR).

2.11. Trypan blue exclusion assay

NIH-3T3 cells were arrested at G0 phase by subjecting them to contact inhibition during culture to confluence. The synchronized cells were released into the cell cycle by splitting the cells onto new dishes. After 2 and 4 days, cells were harvested and stained with trypan blue. Viable cells, which excluded trypan blue dye, were counted in triplicate with a Countess (Invitrogen, Carlsbad, CA).

2.12. Statistical analysis

The results were recorded as the mean ± S.D. Student’s t-test was performed to analyze the differences in absorbance at 450 nm/620 nm, which represented cell growth. A P-value of less than 0.01 was considered statistically significant in all experiments.

3. Results

The NIH-3T3 cells showed density inhibition and subsequent ST2 gene induction by growth stimulation as reported previously for BALB/c-3T3 cells (Fig. 1) [1,2,18]. To determine whether
continuous expression of ST2 or ST2L affects the activation of NIH-3T3 cell proliferation, we constructed NIH-ST2, NIH-ST2L, and control NIH-MSCV cells as described in Section 2. The continuous overexpression of ST2 and ST2L mRNAs in NIH-ST2 and NIH-ST2L cells, respectively, was confirmed (Fig. 2A). The corresponding protein products were detected in the cell lysates (Fig. 2B). Importantly, ST2 protein was detected in the culture medium of NIH-ST2 cells, but not in those of NIH-MSCV and NIH-ST2L cells.

The cell proliferation assay was performed according to the previous report [17]. Continuous expression of ST2 enhanced cell growth (Fig. 3A). However, the overexpression of ST2L had no apparent effect on cell proliferation (Fig. 3B). We next examined the effect of conditioned media from NIH-ST2 cells on the proliferation of NIH-3T3 cells. NIH-3T3 cells treated with the medium containing ST2 showed a significantly higher level of growth than those treated with the conditioned medium from NIH-MSCV cells (Fig. 3C). Moreover, the addition of purified ST2 to the culture medium also enhanced cell proliferation (Fig. 3D).

One difficult to interpret result of this study was that the endogenous ST2 gene was induced by growth stimulation, and ST2 protein was excreted even in the control NIH-3T3 cells after the initiation of cell proliferation (Fig. 1) [2]. Therefore, the qualitative difference between NIH-MSCV and NIH-ST2 cells was that ST2 was expressed only in the density-inhibited quiescent state (Fig. 2A).

To overcome the inherent limitations of a mutant overexpression study, we next performed a knockdown experiment. According to the method used to explore candidate sequences, 3 possible sequences for shRNA construction, located in the common region between ST2 and ST2L, were proposed, and the vectors and resulting transformed cells were named KD1, 2, and 3, respectively, as described in Section 2. Unfortunately, no appropriate sequence specific to ST2 or ST2L was proposed. Although the knockdown process did not work in KD1 cells (data not shown), we were able to confirm that both the ST2 and ST2L expressions were suppressed in KD2 and KD3 cells, and the vectors and resulting transformed cells were named KD1, 2, and 3, respectively, as described in Section 2. Unfortunately, no appropriate sequence specific to ST2 or ST2L was proposed.

Because the expressions of both ST2 and ST2L were reduced in KD2 and KD3 cells, the next question was which was responsible for the observed enhancement of cell growth. To address this, we attempted to express the shRNA-resistant ST2 or ST2L in cells in which the ST2 gene products were reduced, and then observed the effects on cell proliferation.

As shown in Fig. 4B, we constructed vectors carrying mutated

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**Fig. 4.** Knockdown of the ST2 gene products and expression of shRNA-resistant ST2 and ST2L in KD2 cells. (A) The knockdown procedure was carried out as described in Section 2, and quantitative PCR was carried out as in Fig. 1B. (B) Native (nat) and shRNA-resistant (mut) nucleotide sequences from nucleotides number 876 to 894 [2] were aligned along with corresponding amino acid sequence in the middle. Exchanging the 3rd letter of each of 5 codons did not alter the resultant amino acid sequence. (C) The KD2 cells introduced with shRNA-resistant ST2 and shRNA-resistant ST2L expression vectors containing mutant sequences (mut in Panel B) were designated KD2RST2 and KD2RST2L, respectively. Quantitative PCR was carried out as in Fig. 1B. (D) FLAG-tagged shRNA-resistant ST2 and ST2L proteins in the conditioned media (CM) and whole cell lysate (WCL) of the infected NIH-3T3 cells were detected by immunoblotting with M2 antibody.
sequences in which the 3rd nucleotide in each of 5 codons in the target sequence was exchanged in order to avoid degradation of mRNA, while still encoding the same protein product from native mRNA. Then the additional infection of retroviruses encoding shRNA-resistant ST2 and ST2L was performed, and the resultant cells were named KD2RST2 and KD2RST2L, respectively. Indeed, the expressions of the mRNA and protein product for ST2 were recovered in KD2RST2 cells, and the expressions of the mRNA and protein product for ST2L were recovered in KD2RST2L cells, while both were reduced in KD2 cells (Fig. 4C,D).

The cell growth was recovered to a level similar to that in the control when the expression of the ST2 protein was restored by supplementation with the ST2-producing vector (Fig. 5C). On the other hand, supplementation with the ST2L-producing vector had no apparent effect on the cell growth (Fig. 5D). Taken together, these results showed that ST2 promoted the growth of NIH-3T3 cells.

4. Discussion

The key point in the present experimental design was the introduction of fibroblastic cells into a quiescent state in which endogenous ST2 gene expression was scarcely detected (see Fig. 1 and [2]), before starting the cell proliferation assay. As described in the Introduction, the ST2 gene was originally identified as a gene induced in the course of the initiation of cell proliferation of quiescent BALB/c-3T3 cells [1,2]. In our present experiments, we similarly observed the serum-induced expression of ST2 gene products in NIH-3T3 cells. NIH-3T3 cells are the most useful cell line for analysis of the cell cycle, because they can be easily induced into G0 phase by cell density growth inhibition [17,18]. Since the ST2 gene is continuously expressed throughout the cell growth cycle [2], it is difficult to determine the effect of adding ST2 in cycling cells. Indeed, no remarkable growth-enhancing effects of ST2 were detected in our preliminary experiments using continuously growing NIH-3T3 cells (data not shown).

Recently, we found that IL-33 had a dual function in the cell proliferation of NIH-3T3 cells [17]. In addition, we previously reported that ST2 inhibited the IL-33–ST2L signaling pathway by capturing IL-33 in media [5], and thus the relationship between
ST2 and IL-33 is of great interest. We therefore examined this relation in a batch of preliminary experiments, but we found no positive or negative effect of IL-33 on the enhancement of cell proliferation of NIH-3T3 by the addition of ST2. Pre-treatment with IL-33 also did not influence the ST2-induced promotion of cell proliferation (data not shown) [17]. At present, it appears that ST2 itself may be responsible for the growth enhancement of NIH-3T3 cells, via an as-yet-unknown cell surface receptor for ST2 or via direct internalization as reported previously [16]. Indeed, the unexpectedly remarkable effect of shRNA-resistant ST2 (Fig. 5C) suggested that the final target of the ST2 action may be inside the cells.

Knockdown strategy for ST2L has essential limitations. In part, this is because a major function of ST2L is receptor activity in collaboration with IL-1RAcP to transduce IL-33 signals into the cellular interior. Generally, a small amount of receptor molecule on the cell surface is sufficient for signal transmission, as reported previously for IL-1 receptors [22,23]. The ST2L molecules remaining after the knockdown procedure might have been adequate for signal transduction, with the result that no obvious effects of ST2L supplementation were detected. At least, we showed that over-expression of ST2L had no effect on the growth of NIH-3T3 cells (Fig. 3B). In addition, ST2-deficient mice have been shown to be defective in the development of Th2-like cytokine responses, but are not lethal in terms of ontogenesis [24]. Since both ST2 and ST2L are defective in ST2-deficient mice, some redundancy might be at play, which would suggest that ST2 and/or ST2L are not indispensable for cell growth itself.

Since the ST2 gene is induced in the course of initiation of cell proliferation, from G0 to G1/S in an all-or-none fashion [2], the possibility of a positive feedback system in cell growth regulation is of great interest and would be worth investigating in relation to the etiology and prognosis of various diseases. Indeed, as for malignancies, there are several reports suggesting positive or negative effect of IL-33–ST2/ST2L axis on cell growth, cell migration, and anchorage-independent cell growth [15,25–27]. We have no suitable explanation for this discrepancy about the differences of effect that ST2 exhibits against tumor growth. However, our current research is the first case, which shows the function of ST2 protein stimulating cell growth of un-transformed fibroblast. Our simple experimental system presented here may be helpful to enhance such investigations.

Author disclosure statement

No competing financial interests exist.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.bbrep.2015.11.020.

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