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Thrombospondin-1 Inhibits Interleukin-10 Release from Monocytic Cells through Interaction with CD47

Yasushi YAMAUCHI, Motomu KUROKI*, Takayuki IMAKIIRE*, Koichi UNO*,
Yuichi YAMASHITA, Takayuki SHIRAKUSA and Masahide KUROKI*

*Second Department of Surgery and *Department of Biochemistry,*
Fukuoka University School of Medicine

Abstract: Interleukin-10 (IL-10), an immunosuppressive cytokine which plays a key regulatory role in immune responses, modulates inflammation, wound healing, and tumor growth. We have recently shown that thrombospondin-1 (TSP-1), a multifunctional extracellular matrix protein, inhibits the release of IL-10 from human monocytic cells by activating transforming growth factor-β_1 (TGF-β_1). We herein demonstrate an additional, novel inhibitory mechanism of IL-10 release by TSP-1. Human monocytic U937 cells were stimulated *in vitro* with phorbol myristate acetate and LPS in the presence of immobilized TSP-1 or proteolytic 70-kD fragments of TSP-1. The concentration of IL-10 in culture supernatants was determined by an enzyme-linked immunosorbent assay. Both TSP-1 and the 70-kD fragment inhibited the release of IL-10 from the U937 cells. Although the specific sequence to activate TGF-β_1 exists in both molecules, intact TSP-1 revealed a stronger inhibitory effect than the 70-kD fragment at the same molar concentration. CD47 engagement by anti-CD47 antibody or the 4N1K peptide, which corresponds to the CD47-binding site present on TSP-1 but not on the 70-kD fragment, also attenuated the release of IL-10. However, the CD47 engagement with anti-CD47 did not induce any TGF-β_1 release and the RGDS peptide did not affect the IL-10 release, thus suggesting that the inhibition of IL-10 release via CD47 is neither associated with TGF-β_1 nor dependent on $\alpha v \beta 3$ integrin. CD47 engagement by TSP-1 is therefore considered to be a novel pathway to downregulate IL-10 release from monocytic cells.

Key words: CD47, interleukin-10 (IL-10), transforming growth factor-β_1 (TGF-β_1), thrombospondin-1 (TSP-1), U937 cells

Introduction

Thrombospondin-1 (TSP-1), an extracellular matrix glycoprotein, has been shown to exhibit many biological activities, including the modulation of cell adhesion, migration, and proliferation, as well as the inhibition of angiogenesis and tumor growth (reviewed by Sargiannidou et al.[1]). TSP-1 consists of several domains that specify distinct functions through their interaction with a variety of cell surface molecules, such as integrins, CD36,[2] CD47,[3] low density lipoprotein recep-

Correspondence to: Motomu KUROKI, Ph. D., Department of Biochemistry, Fukuoka University School of Medicine, 7-45-1 Nanakuma, Jonan-ku, Fukuoka 814-0180, Japan.
Tel: +81-92-801-1011 Fax: +81-92-801-3600. E-mail: mokuroki@fukuoka-u.ac.jp.

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tor－related protein[4] and pro-teoglycans.[5] Recently, accumulated data indicate that TSP－1 plays a broad role in the immune system:[3)4)6)] TSP－1 selectively inhibits IL－12 production by monocytes through the CD47－transmitted activation of phosphoinositide 3－kinase (PI3－K)[3] and it also increases the macrophage recognition of neutrophils undergoing apoptosis by cooperating with CD36 and $\alpha v \beta 3$ integrin.[2]

Interleukin－10 (IL－10), an immunosuppressive cytokine, plays a key role in regulating immune responses and tolerance by modulating the differentiation and functions of various hematopoietic cells, including T cells, B cells and macrophages (reviewed by Moore et al.[7]). IL－10 also modifies tumor growth and metastasis by affecting the immune system and tumor angiogenesis, and hence the effective immunotherapy of cancer with IL－10 is feasible.[8)－10)] Although TSP－1 is generally accepted to be an immunosuppressive agent,[2)3)11)12)] we have recently shown that TSP－1 may act as an immunopotentiating agent in a phase of the immune response where macrophages play an important role, by demonstrating that TSP－1 inhibits the release of IL－10 from human monocytic U937 cells by activating transforming growth factor－β_1 (TGF－β_1).[13] In the present study, we further investigated the regulatory mechanism of IL－10 release from U937 cells by TSP－1, and found an additional pathway through which TSP－1 downregulates the release of IL－10 by directly interacting with CD47 on such cells.

Materials and Methods

Peptides and reagents: The 4N1K peptide (KRFYVVMWKK) derived from the carboxy－terminal domain of TSP－1 and its control peptide 4NGG (KRFYGGMWKK) were obtained from Sigma Genosys (Ishikari, Japan). The RGDS peptide derived from the type 3 repeats of TSP－1, its control peptide RGES, phorbol myristate acetate (PMA), LPS, human fibronectin and type Ⅳ collagen were all purchased from Sigma Chem Co. (St. Louis, MO).

Antibodies: Two monoclonal antibodies (MAbs) against CD47, B6H12 and BRIC126, were from PharMingen (San Diego, CA) and SANBIO (AM Uden, Netherlands), respectively. The nonspecific control IgG MOPC21 was from Sigma. MAbs against human IL－6 (5IL6) and IL－10 (9D7), and biotinylated MAbs for IL－6 (7IL6) and IL－10 (12G8) were obtained from ENDOGEN (Woburn, MA).

Purification of intact TSP－1 and the 70－kD proteolytic TSP－1 fragment: TSP－1 was purified by affinity chromatography using heparin－ and fibrinogen－Sepharose columns from human platelets activated with calcium ionophore according to a previously described method.[14] To remove the TGF－β_1 associated with TSP－1 from the preparation, we applied a TSP－1 sample on gel filtration with a Superdex 200 column (Amersham Pharmacia Biotech, Uppsala, Sweden) at pH 11.0 in 0.01 M Tris－buffered saline (TBS) as previously described.[15] TSP－1 was then dialyzed against TBS, pH 7.4, containing 2 mM $CaCl_2$. The 70－kD proteolytic TSP－1 fragment, which contains the type 1 and type 2 repeats, was generated according to a previously described method.[16] Briefly, intact TSP－1 was digested with L－1－tosylamido－2－phenylethylchloromethyl ketone－treated chymotrypsin (Sigma; 1:100 w/w) in the presence of 10 mM EDTA in TBS, pH 7.4, for 30 min at room temperature and the 70－kD fragment was then isolated by gel filtration. The concentrations of TSP－1 and the 70－kD fragment were determined by a protein assay kit (Bio－Rad, Hercules, CA).

Cell culture: 937 cells[17] were obtained from the Japanese Research Resource Bank (Tokyo, Japan) and they were grown in suspension in RPMI－1640 medium (Sigma) containing 10% fetal calf serum, 100 U/ml of penicillin and 100 μg/ml of streptomycin at 37℃ in a 5% CO_2 humidified incubator. The cells were plated at 1×10^6/ml in 96－well, flat－bottom culture plates (Corning, Corning, NY) and they were stimulated with phorbol myristate acetate (PMA) (1 ng/ml) and LPS (10 μg/ml) for 48 h. For the immobilization of TSP－1 and the 70－kD fragment onto the plates, each protein dissolved in Dulbecco's phosphate－buffered saline (PBS) was added to the wells of the plates (100 μl/well),

which were then incubated overnight at 4℃.

Measurement of cytokines: IL-10 and IL-6 in spent culture media were quantitated by sandwich-type enzyme-linked immunosorbent assays (ELISA) as described previously.[13] Briefly, 96-well ELISA plates (Greiner, Frickenhausen, Germany) were incubated with 100 μl/well of PBS containing 2 μg/ml anti-IL-10 MAb or anti-IL-6 MAb overnight and then were blocked by incubation with PBS containing 2% BSA. The wells were serially incubated with spent culture media, biotinylated antibody for IL-10 or IL-6, and horseradish peroxidase-conjugated avidin before undergoing color development. The standard curves were obtained with recombinant human IL-10 and IL-6 (ENDOGEN). The measurement of TGF-β_1 was performed using an ELISA kit (R & D Systems, Minneapolis, MN) according to the manufacturer's protocol.

Statistical analysis: The results were compared using the two-tailed Student's t-test. All values presented are the means ± S.D. A P value of <0.05 was considered to be significant.

Results

TSP-1 selectively inhibits the release of IL-10 from the stimulated U937 cells: As shown previously,[13] the U937 cells released IL-10 into the culture medium by stimulation with PMA and LPS (Fig. 1A). When the cells were cultured in the wells coated with 55 nM TSP-1, IL-10 release was significantly reduced, whereas two other extracellular matrix proteins, fibronectin and type Ⅳ collagen, did not significantly affect the release of IL-10. This inhibitory effect of TSP-1 on IL-10 release was dependent on the concentration of the TSP-1 used for immobilization onto the plate and a plateau was reached at 100 nM (Fig. 1B).

Comparison of the inhibitory effects of TSP-1 and its proteolytic 70-kD fragment: TSP-1 comprises several different domains, each of which is responsible for the different functions of TSP-1.[16)18)19)] To determine the structure of TSP-1 essential for the inhibitory effect of TSP-1, we compared the 70-kD proteolytic fragment of TSP-1 with the intact TSP-1 for the effect on IL-10 release. The fragment contains the type 1 and type 2 repeats of TSP-1 but lacks the N-terminal heparin-binding domain, the RGD sequence,

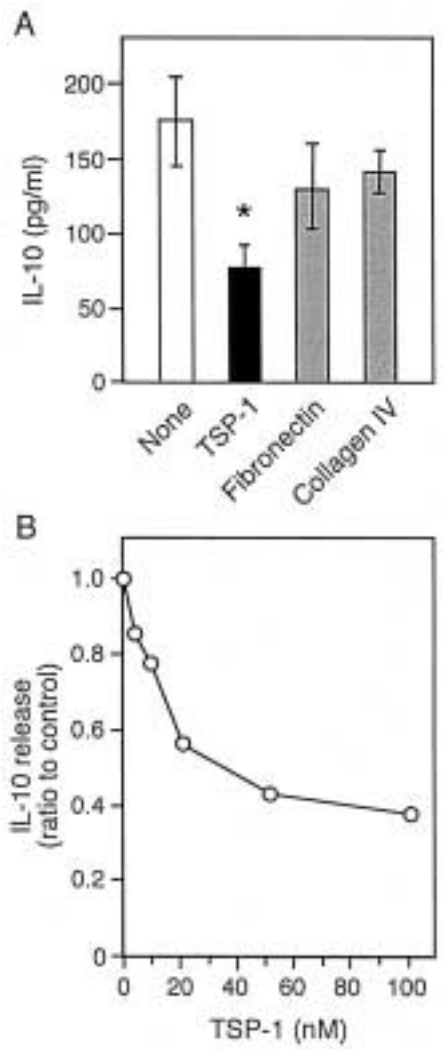

Fig. 1. Effect of immobilized TSP-1 on the IL-10 release from the U937 cells. The U937 cells were stimulated for 48 h with or without PMA and LPS in the wells coated with 55 nM of TSP-1, fibronectin, collagen Ⅳ (A) or with different concentrations of TSP-1 (B). IL-10 in spent culture medium was quantitated by ELISA. The results represent the means ± S.D. ($n=3$) (A) and the mean values of duplicate cultures (B). *, $P<0.05$.

and the C-terminal CD47-binding domain.[16] As shown in Fig. 2, the immobilized 70-kD fragment continued to have a suppressive effect of intact TSP-1 on IL-10 release, although the inhibitory effect of the 70-kD fragment was significantly weaker than that of the intact TSP-1 at the molar concentration that exhibited maximal inhibition (100 nM). We have previously demonstrated that TGF-β_1 activated by TSP-1 inhibits the release of IL-10.[13] The type 1 repeats within the 70-kD fragment contain RFK and WSXW motifs, which are involved in the conversion of inactive latent TGF-β_1 (L-TGF-β_1) to active mature TGF-β_1.[20] The inhibition of IL-10 release by the 70-kD fragment could therefore be partly attributed to the presence of TGF-β_1 activated by the RFK and WSXW motifs. However, the lower inhibitory activity of the 70-kD fragment than that of the intact TSP-1 molecule suggested the possibility that, in addition to the TGF-β_1 activating sequences, a structure present on intact TSP-1 but absent from the 70-kD fragment may also be responsible for the inhibition of IL-10 release.

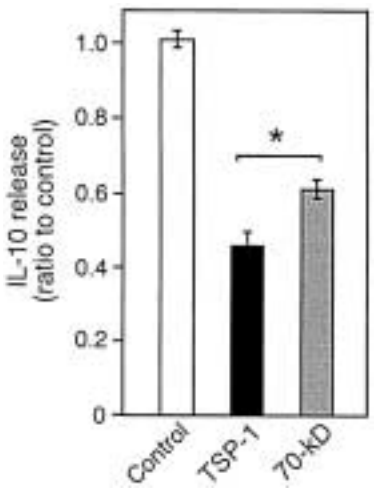

Fig. 2. Comparison of inhibitory effects of TSP-1 and its 70-kD fragment on IL-10 release from the U937 cells. The U937 cells were incubated with PMA and LPS for 48 h in the wells uncoated (control) or coated with 100 nM TSP-1 or the 70-kD fragment. IL-10 in the spent culture medium was quantitated by ELISA. The means ±S.D. are shown (n=3). *, $p<0.05$.

CD47 engagement selectively inhibits IL-10 release from the U937 cells: From the results described above, we hypothesized that the interaction of cell surface CD47 with the CD47-binding domain of TSP-1, which is absent from the 70-kD fragment, is responsible for the additional inhibition of IL-10 release by intact TSP-1. CD47 engagement by immobilized anti-CD47 MAb (Fig. 3A) or the soluble 4N1K synthetic peptide (Fig. 3B), which corresponds to the CD47-binding site of TSP-1,[6] attenuated the release of IL-10, whereas the release of the proinflammatory cytokine IL-6 was not affected by the ligation of CD47 (Figs. 3D and 3E).

The inhibition of IL-10 release via CD47 is independent of TGF-β_1 and $\alpha_v\beta_3$ integrin: Ligation of $\alpha_v\beta_3$ integrin can result in the production of TGF-β_1, a potent inhibitor of proinflammatory cytokine synthesis, by macrophages, possibly because of the interaction between CD47 and $\alpha_v\beta_3$ integrin.[20]

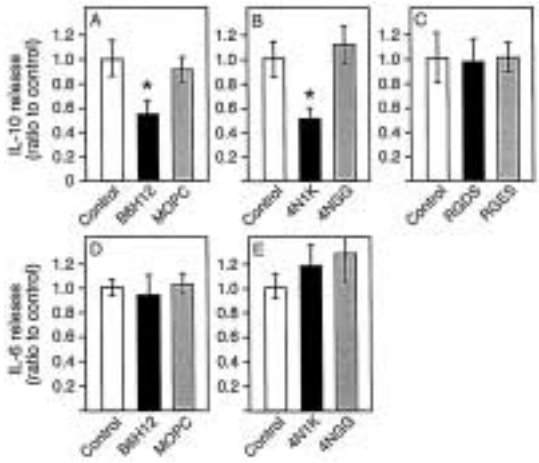

Fig. 3. Suppression of IL-10 release by CD47 engagement. The U937 cells were stimulated for 48 h with PMA and LPS in the wells coated with 10 μg/ml anti-CD47 MAb B6H12 or control MOPC21 IgG (A and D) or in the presence of 100 μM soluble 4N1K, 4NGG (B and E), RGDS or RGES peptide (C). IL-10 (A, B, and C) and IL-6 (D and E) in the spent culture medium were quantitated by ELISA. The means±S.D. are shown (n=3). *, $p<0.05$.

However, the RGDS synthetic peptide derived from the type 3 repeats of TSP-1, which binds to $\alpha v \beta 3$ integrin,[2] never affected IL-10 release (Fig. 3C). Furthermore, CD47 engagement with immobilized anti-CD47 MAb or the 4N1K peptide did not induce the release of TGF-β from the U937 cells (data not shown). Together, these results indicated that CD47 engagement by immobilized MAb or its natural ligand suppressed the release of IL-10 from the stimulated U937 cells through a TGF-β_1- and integrin-independent mechanism.

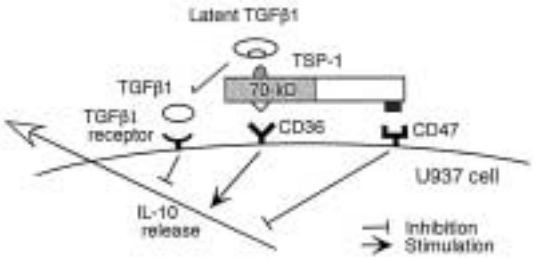

Fig. 4. Schematic illustration of the possible control mechanisms of IL-10 release by TSP-1 through three different pathways.

Discussion

We have previously demonstrated that the activation of TGF-β_1 by the type 1 repeats of TSP-1 is responsible for the inhibition of IL-10 release by TSP-1.[13] In the present study, another mechanism for the inhibition of IL-10 release by TSP-1 was suggested by a comparison of the inhibitory effects of intact TSP-1 and the 70-kD fragment of TSP-1; the 70-kD fragment containing the type 1 repeats showed a significantly weaker inhibitory effect on IL-10 release than intact TSP-1. CD47, a membrane protein also called integrin-associated protein (IAP), is one of the ligands of TSP-1[18] and it is also functionally expressed on the U937 cells.[21] TSP-1 interacts with CD47 through its C-terminal domain[18], which is absent from the 70-kD fragment. We therefore speculated that TSP-1 could affect the release of the cytokine from the U937 cells by interaction with CD47. Indeed, CD47 stimulation, mimicked by the binding of immobilized MAb or soluble 4N1K peptide corresponding to the CD47-binding sequence of TSP-1, significantly inhibited the release of IL-10, but not of IL-6, from U937 cells without affecting TGF-β_1 activation. These findings suggested that a part of the TSP-1-mediated inhibition results from its direct interaction with CD47, and that such CD47-mediated inhibition is not associated with TGF-β_1 activation (see Fig. 4).

The detailed intracellular inhibition pathway of IL-10 release through CD47 after the binding of TSP-1 is not clear at present. Armant and colleagues[3] demonstrated a similar inhibition of IL-12 release from human monocytes by CD47 engagement; this downregulation required the activation of PI3-K, which might also be involved in the present TSP-1-mediated inhibition of IL-10 release via CD47.

There have been many studies demonstrating the physical and functional association between CD47 and $\alpha v \beta 3$ integrin (reviewed by Brown et al.[22]); for example, a ligation of CD47 produces a signal identical to that resulting from a ligation of $\alpha v \beta 3$ integrin.[23] However as shown in the present study, the RGDS synthetic peptide, which corresponds to the $\alpha v \beta 3$ integrin-binding site of TSP-1 within the type 3 repeats, did not affect IL-10 release, suggesting that the inhibition of IL-10 release is an integrin-independent function of CD47. This finding is inconsistent with the notion that CD47 and $\alpha v \beta 3$ integrin are functionally linked, but there is also evidence that CD47 can function in an integrin-independent manner on lymphocytes.[24][25]

Contrary to this inhibitory effect on IL-10 release, the latent ability of TSP-1 to enhance IL-10 release through the interaction of the type 1 repeats with CD36 on U937 cells was suggested in our recent study.[13] The inhibition by intact TSP-1 probably results from a strong inhibition induced by the additive effects of activated TGF-β_1 and CD47 signaling, which overwhelms the augmenting effect of TSP-1 via CD36 (Fig. 4). In our past study on human peripheral blood monocytes[11], an enhancing effect on IL-10 release was demonstrated by TSP-1. This

discrepancy for the regulation of IL-10 release by TSP-1 may be related to cell-specific properties. In addition, it is possible that TSP-1 exerts an inhibitory or enhancing effect on IL-10 release from monocytes or macrophages depending on which domain/fragment is more functional in a certain biological setting, and a differential expression or activation of the cell surface receptors for TSP-1 dictates the specific responses of each cell type to TSP-1.

IL-10 has been reported to limit inflammation by reducing the synthesis of proinflammatory cytokines[26] or by suppressing the function of antigen-presenting cells by down-regulating HLA class I and II molecules.[27] Moreover, the preliminary results for IL-10 therapy in the growth and metastasis of various tumors appear to be promising.[8)-10)]

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