

Original Article

Elevated concentrations of soluble TCTA protein-derived products including an inhibitor of human osteoclastogenesis in peripheral blood of untreated patients with early-onset rheumatoid arthritis

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Abstract

T-cell leukemia translocation-associated gene (TCTA) protein is expressed ubiquitously in normal human tissues, but its function is not completely understood. We have demonstrated that TCTA protein is essential for human osteoclastogenesis induced by NF- κ B ligand (RANKL), inhibits the proliferation of both small-cell lung carcinoma and fibroblast-like synoviocytes in rheumatoid arthritis (RA), and may act as a coupling factor in bone metabolism. In the current study, we constructed a high sensitivity sandwich ELISA system and measured the concentration of soluble TCTA protein-derived products (sTDP), which include an inhibitor of human osteoclastogenesis, in the peripheral blood from two groups of untreated patients with early-onset RA and normal volunteers (NV). In the first group, the concentration of sTDP was significantly higher in the 4 RA patients than in the 3 NV ($p=0.0339$). The level of C-reactive protein (CRP) in serum was not correlated with the concentrations of sTDP in the RA patients. We confirmed these results by analyzing the second group, which was comprised of 22 untreated patients with early-onset RA and 5 NV. In conclusion, sTDP levels were elevated in untreated patients with early-onset RA independent of inflammation. These findings suggest that sTDP play an important role in the pathogenesis of RA by inhibiting both osteoclastogenesis and the proliferation of fibroblast-like synoviocytes.

(Key Words: osteoclast, rheumatoid arthritis, T-cell leukemia translocation-associated gene (TCTA) protein)

Introduction

In 1995, Aplan *et al.* cloned and characterized a novel gene at the site of a t (1:3) (p34;p21) translocation breakpoint in T-cell acute lymphoblastic leukemia, designating this gene as TCTA 1. TCTA protein and mRNA are expressed ubiquitously in normal human tissues¹. In that study, genomic Southern blots demonstrated a reduced TCTA signal in three out of four small cell lung cancer cell lines, suggesting the loss of one of the two gene copies¹. It was later reported that TCTA protein interacts with SMA- and MAD-related protein 4 (SMAD4)². However, the function of TCTA protein was not clarified until 2009, when we revealed its role in human osteoclastogenesis³.

In 2009, we identified a novel peptide, GQN, derived from the extra-cellular domain of TCTA protein, inhibits both the RANKL-induced differentiation of human osteoclasts (Oc) and the pit formation of mature human Oc *in vitro*³. We also showed that TCTA protein is expressed on human monocytes. The 29-mer peptide GQNGSTPDGSTHFPSEWEMAANEPLKTHRE from TCTA protein, which we named "peptide A", most potently inhibited the differentiation of human Oc³. Thus, we hypothesized that the interaction of TCTA protein and its counterpart protein plays an essential role in the differentiation of human Oc and that the interaction is inhibited by peptide A. In addition, we have demonstrated

that peptide A from TCTA protein inhibits the proliferation of small lung cells^{4,5} and human synovial fibroblast-like cells from patients with rheumatoid arthritis (RA)⁶.

We also performed mouse experiments *in vivo*. We generated mouse peptide A and two other peptides including GQN from the mouse sequence of TCTA. However, these peptides did not inhibit the differentiation of mouse Oc from mouse bone marrow cells *in vitro*³. In contrast, human peptide A inhibited the differentiation of primate Oc from peripheral monocytes from crab-eating monkeys⁷.

To clarify the role of TCTA protein in bone metabolism *in vivo*, we generated systemic and Oc-specific TCTA gene transgenic (Tg) mice and conditional knock-out mice and analyzed their bones⁸. We hypothesized that in Tg mice with over-expressed TCTA protein, the bone volume decreases with activated osteoclastogenesis (Hypothesis 1). We also hypothesized that in conditional knockout mice with TCTA protein-deficient Oc, the bone volume increases with inhibited osteoclastogenesis (Hypothesis 2). Our findings completely supported Hypothesis 1, but not Hypothesis 2. According to these findings, we speculated that TCTA protein expressed on Oc acts as a coupling factor, which is defined as a factor produced by Oc to induce the differentiation of osteoblasts⁹⁻¹³.

In the current study, to measure the concentration of soluble TCTA protein-derived products (sTDP), which include peptide A, in the peripheral blood of untreated patients with early-onset RA, we constructed a high sensitivity sandwich ELISA system. Both polyclonal and monoclonal antibodies were generated. We also analyzed the correlation between the levels of sTDP in the peripheral blood of untreated patients with early-onset RA and the clinical characteristics, such as the levels of serum CRP, anticyclic citrullinated peptide antibody (ACPA), and rheumatoid factor (RF).

Patients and Methods

Profiles of Patients

We analyzed two different groups. The first group

included 4 female untreated patients with early-onset RA who met the American College of Rheumatology (ACR) 1987 revised classification criteria and 3 NV who were treated as controls. The disease duration was less than 7 months for all patients (Table 1). The patients had not been treated by DMARDs or corticosteroids when peripheral blood was obtained.

The second group included 22 untreated patients with early-onset RA, including 18 ACPA-positive patients, and 5 NV who were treated as controls. All patients met the American College of Rheumatology (ACR) 1987 revised classification criteria, and the disease durations was less than 12 months for all patients. The patients had not been treated by DMARDs or corticosteroids when peripheral blood was obtained.

Plasma was obtained from peripheral blood using heparinized tubes. Ages and genders were not significantly different between the RA patients and NV (data not shown).

The current study was approved by the ethical committee of Tokyo Women's Medical University (#3651). Informed consent was obtained from each patient.

Production of rabbit polyclonal antibody against human TCTA protein

The method for the production was explained previously³. One peptide, CEPLKTHRE (MW11123) was synthesized and conjugate to keyhole limpet hemocyanin (KLH). Two rabbits were immunized with CEPLKTHRE-KLH conjugate 4 times at 2-week intervals. The whole antisera were collected at day 56; 2 sera were designated as #1 and #2. The values of antisera were 73ml and 72ml, respectively. These antisera were purified using a column. Protein A-Sepharose 4 fast flow (Amersham-Biosciences). The final weights of protein in the 2 antisera, #1 and #2 were 220 mg and 111 mg, respectively³.

Production of mice monoclonal antibody against human TCTA protein

Two peptides, C-GQNGSTPDGSTHFPSEMAANEPLKTHRE (30-mer peptide)-KLH and EPLKTHRE (8-mer peptide)

Table 1. Profiles of patients

patient #	sex	age (year)	diagnosis 2010ACR /EULAR	Disease duration (months)	Mens.	CCP U/mL	RF IU/mL	CRP mg/d	TRAcP5b mU/dL	BAP µg/L	Treatment after the analysis
1	F	54	RA	2	-	-	-	1.5	381	8.4	MTX 6 mg/w
2	F	30	RA	3	+	12.3	26	0.27	305	9.4	MTX 6 mg/w -> 10 mg/w
3	F	67	RA	7	-	225	215	0.99	577	18.2	MTX 6 mg/w
4	F	63	RA	1.5	-	-	-	7.55	480	10.2	MTX 6 mg/w

TRAcP5b (120-420 mU/dL), BAP (premenopausal. 2.9-14.5 µg/L, postmenopausal. 3.8-22.6 µg/L)

Mens., menstruation.

were synthesized. Five Balb/c mice were immunized with the 30-mer peptide conjugated with KLH 6 times over 9-21 days. Splenocytes from the mice were fused with cells from the mouse myeloma cell line P3U1. Finally, two hybridomas, 1F10 and 1E5, producing monoclonal antibodies were obtained. We confirmed that these monoclonal antibodies combined both the 8-mer peptide and 30-mer peptide. This finding confirmed that the epitopes of these antibodies were included in EPLKTHRE, which is the common part of both peptides. 1F10 was deposited at the National Institute of Technology and Evaluation (NITE) Patent Microorganisms Depository (NPMD) (# NITE P-01747, 1F10).

Development of a novel sandwich ELISA system with high sensitivity to detect sTDP

A NUNC Maxisorp plate (ThermoFisher Scientific, Waltham, MA, USA) was coated with monoclonal antibodies from 1F10. Plasma from patients, NV and a standard were added to the plate, and sTDP was bound to capture the antibodies. Antisera #2 was purified by Protein A affinity chromatography and biotinylated with Biotin Labeling Kit-NH2 (Dojindo Molecular Technologies, Inc., Kumamoto, Japan). Biotin-conjugated antisera #2 was added to the plate and bound to sTDP. Bound biotinylated antisera #2 was probed with streptavidin-horseradish peroxidase (Biolegend, Inc., San Diego, CA, USA) (Figure 6). Chromogenic reactions were proceeded with a 3, 3', 5, 5'-Tetramethylbenzidine set (Biolegend, Inc.) for 30 minutes and stopped by adding 2 M H₂SO₄. The absorbance was measured using a BIO-RAD plate reader at 450 nm (Bio-Rad, Hercules, CA, USA). Recombinant human TCTA (Proteintech, Rosemont, IL, USA) was used as the standard.

Statistical analysis

Data were analyzed using the Wilcoxon test and Spearman's test (StatView®; Abacus Concepts Inc., Berkeley, CA). Significant differences are defined as $p < 0.05$.

Results

Concentration of sTDP in the first group of patients

The concentration of sTDP was significantly higher in RA patients than in normal volunteers (NV) ($p=0.0339$) (Figure 1A).

Correlation of sTDP and clinical data in the first group of patients

The levels of CRP in serum did not correlate with the concentrations of sTDP in RA patients (Figure 1B).

Concentration of sTDP in the second group of patients

The concentrations of sTDP were significantly higher in RA patients than in NV ($p=0.0372$) (Figure 2A).

Correlation of sTDP and clinical data in the second group of patients

The levels of CRP, ACPA, and RF in serum did not correlate with the concentrations of sTDP in RA patients (Figure 2B, C, D).

The findings in the first group were confirmed by analyzing the second group, which was comprised of 22 untreated patients with early-onset RA and 5 NV.

Discussion

In 1995, Aplan *et al.* cloned and characterized a novel gene at the site of a t(1:3)(p34;p21) translocation breakpoint

Figure 1A

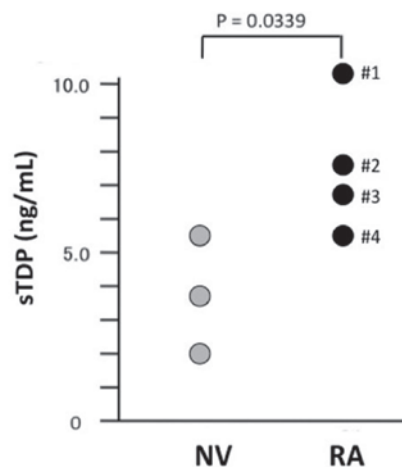


Figure 1A. Concentrations of sTDP in plasma from the first group.

Black circles, RA patients; Gray circles, NV.

Figure 1B

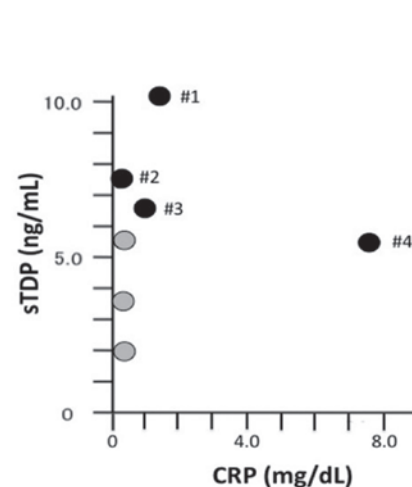


Figure 1B. Correlation between the levels of CRP and concentrations of sTDP in the first group.

Black circles, RA patients; Gray circles, NV.

in T-cell acute lymphoblastic leukemia, designating this gene as TCTA¹. In 2009, we identified a novel peptide, GQN, derived from the extra-cellular domain of TCTA protein, inhibits both the RANKL-induced differentiation of human osteoclasts (Oc) and the pit formation of mature human Oc *in vitro*³.

In the current study, we demonstrated that the levels of sTDP were elevated in the plasma of untreated patients with early-onset RA (Figure 1A, 2A). It is speculated that sTDP play a role in inhibiting osteoclastogenesis, because sTDP include peptide A, which potently inhibits human osteoclastogenesis and the pit formation of mature human Oc *in vitro*³ (Figure 3, 4).

In addition, peripheral sTDP may reach the synovial

tissues and inhibit the proliferation of fibroblast-like synovial cells, because we previously reported that peptide A inhibits the proliferation of fibroblast-like synovial cells (FLS) from patients with RA⁶ (Figure 4).

Since we reported that Peptide A inhibits human osteoclastogenesis and the cell proliferation of FLS form RA, it is possible that sTDP play an important role in regulating both bone destruction and the proliferation of (FLS).

In addition, the levels of sTDP were not correlated with the level of CRP (Figure 1B, 2B), suggesting that the mechanism of sTDP production in RA is independent of inflammation. TCTA protein is expressed ubiquitously in human tissues and cells¹. However, the mechanism by which TCTA protein is cleaved from the membrane is

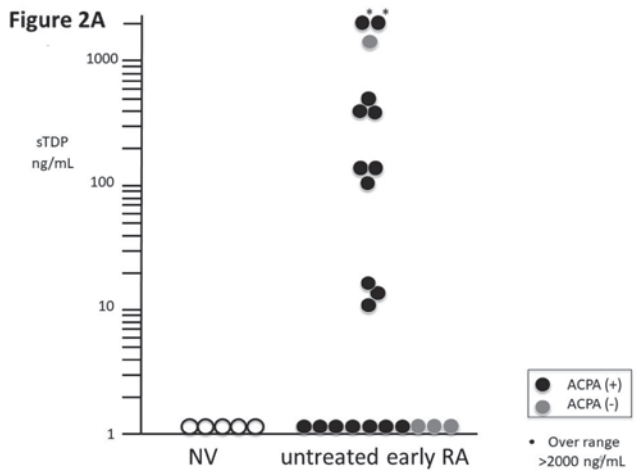


Figure 2A. Concentrations of sTDP in plasma from RA and NV in the second group.

Black circles, ACPA-positive RA patients; Gray circles, ACPA-negative RA patients; White circles, NV.

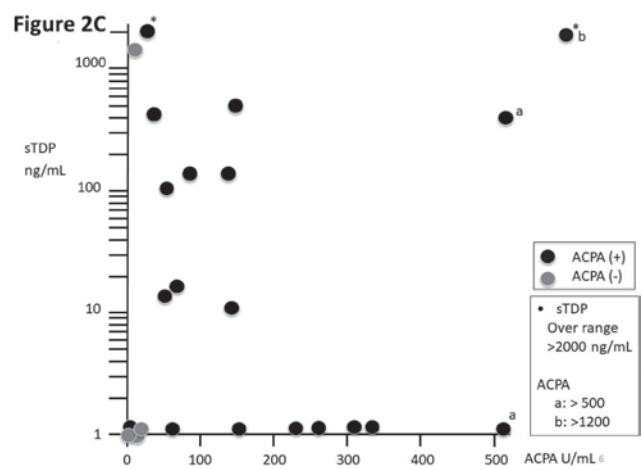


Figure 2C. Correlation between levels of ACPA and concentrations of sTDP in the second group.

Black circles, ACPA-positive RA patients; Gray circles, ACPA-negative RA patients.

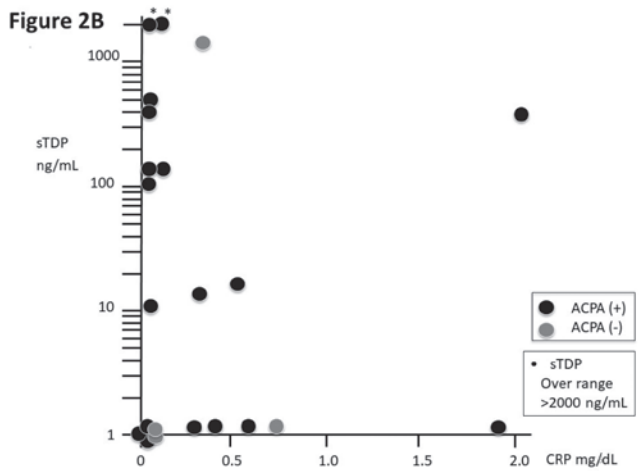


Figure 2B. Correlation between levels of CRP and concentrations of sTDP in the second group.

Black circles, ACPA-positive RA patients; Gray circles, ACPA-negative RA patients.

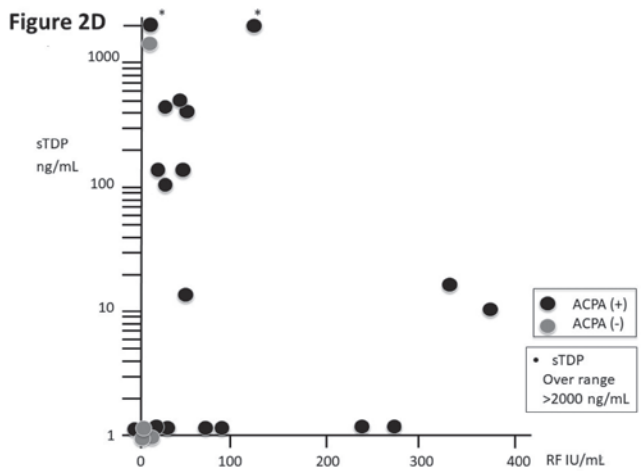


Figure 2D. Correlation between levels of RF and concentrations of sTDP in the second group.

Black circles, ACPA-positive RA patients; Gray circles, ACPA-negative RA patients.

unknown. In 2005, we demonstrated that human Th1 cells expressing RANKL induce human osteoclastogenesis and that these cells do not decrease in number after the disease condition improves, suggesting that the mechanism of osteoclastogenesis is independent of inflammation¹⁴. These findings further indicate that the bone metabolism in RA is regulated by both inflammation and non-inflammation mechanisms.

Elevated levels of sTDP in the peripheral blood of RA patients may effect the differentiation of not only Oc but also osteoblasts. In 2017, we reported the possibility that TCTA protein plays a role in the differentiation of osteoblasts as a coupling factor⁸ (Figure 5). However, it remains unclear whether sTDP derived from TCTA protein effect the

coupling and directly induce or reduce the differentiation of osteoblasts (Figure 5). Further investigation is necessary to resolve this issue.

sTDP is one of only a few factors that inhibit osteoclastogenesis in the pathogenesis of RA. Synovial tissues of patients with RA produce many factors to induce osteoclastogenesis, including RANKL^{7, 15-19}, IL-6²⁰, TNF α ^{21, 22}, IL-17²³⁻³¹, IL-23³², IL-35³³ and IL-1 β ³⁴. In contrast, a small number of factors that reduce osteoclastogenesis, such as osteoprotegerin (OPG)³⁵, IL-4³⁶ and IFN γ ³⁷, are produced by the synovial tissues of RA patients, although we have demonstrated that human Th1 cells expressing both IFN γ and RANKL induce human osteoclastogenesis¹⁴. Ziolkowska et al. reported high levels of OPG in the serum of RA patients, but that these levels were normalized after anti-TNF α treatment³⁵. Raza et al. reported that early-onset RA is characterized by a distinct and transient synovial fluid cytokine profile, which includes IL-4³⁶. Thus, it is speculated that sTDP play an important role in inhibiting

Figure 3

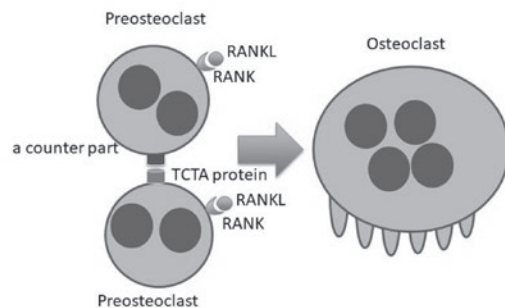


Figure 3. The role of TCTA protein in human osteoclastogenesis

An interaction of TCTA protein and a putative counterpart is required in human osteoclastogenesis induced by RANKL [3, 5].

Figure 4

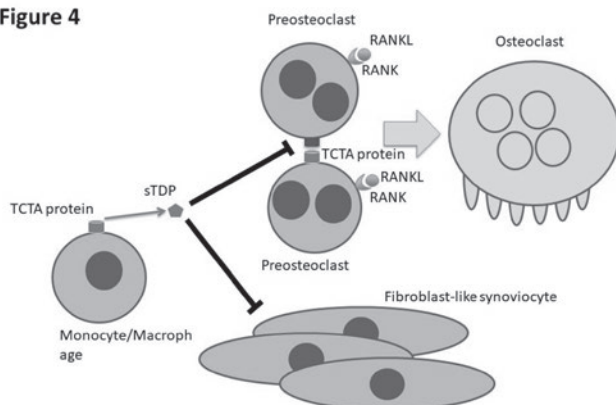


Figure 4. A possible role of sTDP derived from TCTA protein in human osteoclastogenesis and the proliferation of fibroblast-like synoviocytes

sTDP is derived from TCTA protein. sTDP including peptide A inhibit human osteoclastogenesis and the proliferation of fibroblast-like synoviocytes from patients with RA. It is possible that sTDP is derived from preosteoclasts and osteoclasts as well as monocytes/macrophages [3, 5, 6].

Figure 5

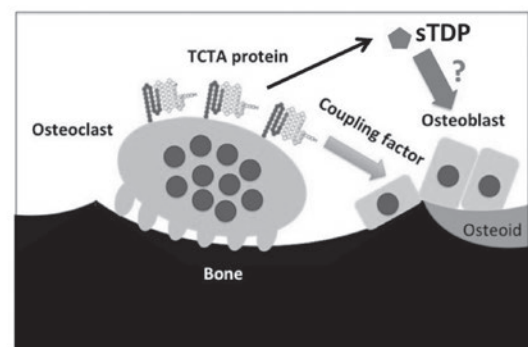


Figure 5. TCTA protein expressed on osteoclasts plays a role as a 'coupling factor' in vivo.

sTDP are derived from TCTA protein. '?' shows that it remains unclear whether sTDP derived from TCTA protein effect the coupling and directly induce or reduce the differentiation of osteoblasts.

Modified from the scheme of ref #8.

Figure 6



Figure 6. Scheme of the novel sandwich ELISA system with high sensitivity to detect sTDP

HRP, horseradish peroxidase.

osteoclastogenesis even though they are not cytokines (Figure 3, 4).

The limitation of the current study is that the number of patients was small. However, it was very difficult to obtain blood samples from untreated patients with early-onset RA, which in this study was defined as a duration of less than 7 months in the first group and 12 months in the second group. We confirmed the results of the first group by analyzing the second group, which included a larger number of untreated early-onset RA patients.

Conclusions

In conclusion, we demonstrated that sTDP levels are elevated in untreated patients with early-onset RA independent of inflammation. These findings suggest that sTDP play an important role in inhibiting both osteoclastogenesis and the proliferation of fibroblast-like synoviocytes in the pathogenesis of RA.

Declaration of interest

Authors declare that there is no conflict of interest regarding the publication of this paper.

Acknowledgments

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未治療早期関節リウマチ患者末梢血中にはヒト破骨細胞分化抑制作用を有する T 細胞性白血病転座関連遺伝子(TCTA)蛋白が上昇している。

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要 約

筆者らはTCTA蛋白がヒト破骨細胞分化に重要であることを見出し報告した。すなわちTCTA蛋白由来ペプチドがreceptor activator of NFkB ligand (RANKL) によるヒトの単球からの破骨細胞形成を抑制することを新たに見出した。今回はsoluble TCTA protein-derived products (sTDP) ELISAシステムを構築した。その結果 1 : sTDPは健常人に比較しRAで上昇していること, これはCRPとは連動しないこと。 2 : 未治療早期RAでは健常人と比較し炎症に関係なく, sTDPが上昇していた。以上よりsTDPは破骨細胞分化抑制と滑膜線維芽細胞分化抑制を制御しRAの病態に関与している事が示唆された。今後新たなRA治療薬となる可能性がある。

(キーワード: 関節リウマチ, 破骨細胞, T細胞性白血病転座関連遺伝子蛋白)