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# The Role of T-Cell Leukemia Translocation-Associated Gene (TCTA) Protein in Human Osteoclastogenesis

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## 1. Introduction

Synovial tissues of patients with rheumatoid arthritis (RA) include factors regulating bone resorption, such as receptor activator NF- $\kappa$ B ligand (RANKL), TNF- $\alpha$ , IL-6, IL-17, and IFN- $\gamma$ . However, in addition to these cytokines, other factors expressed in synovial tissues may play a role in regulating bone resorption. In 2009, we demonstrated that novel peptides from T-cell leukemia translocation-associated gene (TCTA) protein expressed in synovial tissues from patients with RA inhibits human osteoclastogenesis, preventing cellular fusion via the interaction between TCTA protein and a putative counterpart molecule. Only a few studies on the role of TCTA protein have been reported. In the current review paper, we summarized papers on TCTA protein and our recent findings.

Synovial tissues of patients with rheumatoid arthritis (RA) include factors regulating bone resorption by expressing cytokines such as RANKL, TNF $\alpha$ , IL-6, IL-17, and IFN $\gamma$  (Horwood et al. 1999; Kawai et al. 2006; Kobayashi et al. 2000; Kotake et al. 1996; 1999; 2001; 2005; Takayanagi et al. 2000; Yago et al. 2007, Yago et al. 2009). In addition to these cytokines, however, other factors expressed in synovial tissues may play a role in resorbing bone. To identify novel peptides or proteins expressed in synovial tissues of patients with RA that regulate human osteoclastogenesis, we purified proteins from synovial tissues of patients with RA, using gel filtration chromatography, reverse-phase HPLC, and mass spectrometry. We finally demonstrated that a peptide derived from the extra-cellular domain of T-cell leukemia translocation-associated gene (TCTA) protein inhibits both RANKL-induced human osteoclastogenesis and pit formation of mature human osteoclasts (Kotake et al. 2009b, 2009c).

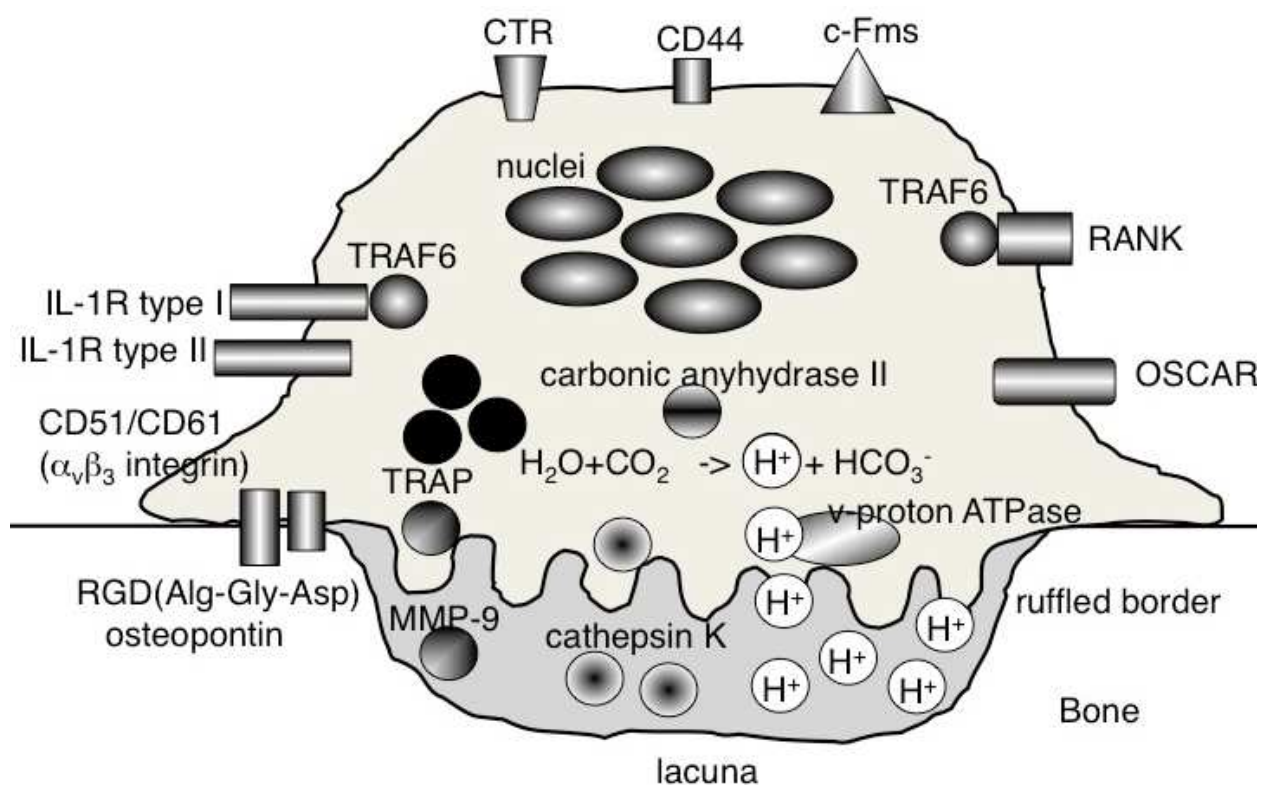
In 1995, Aplan et al. cloned and characterized a novel gene at the site of a t(1;3)(p34;p21) translocation breakpoint in T-cell acute lymphoblastic leukemia, designating this gene as TCTA (Aplan et al. 1995). TCTA is also reported as T-cell leukemia translocation-altered gene. TCTA mRNA is expressed ubiquitously in normal tissues, with the highest levels of expression in the kidney. TCTA has been conserved throughout evolution in organisms ranging from *Drosophila* to humans. A short open reading frame encodes a protein of 103 amino acid residues,  $M_r$  11,300, without strong homology to any previously reported proteins. Of note, genomic Southern blots demonstrated a reduced TCTA signal in three of four small cell lung cancer cell lines, suggesting the loss of one of the two copies of the gene

(Aplan et al. 1995). On the other hand, in 2005, it has been reported that TCTA interacts with SMA- and MAD- related protein 4 (SMAD4) in a proteome-scale map of the human protein-protein interaction network (Rual et al. 2005); however, the function of TCTA has not been clarified.

## 2. Osteoclast

### 2.1 Structure and function of osteoclasts

Osteoclasts are unique multinucleated cells whose specialized function is to resorb calcified tissues (Fig. 1) (Kotake et al. 2005). On the surface of bone, osteoclasts develop a specialized adhesion structure, the 'podosome', which subsequently undergoes reorganization into sealing zones (Luxenburg et al. 2007). These ring-like adhesion structures, i.e., actin rings, seal osteoclasts to the surface of bone. In the sealed resorption lacuna, localized acidification is driven by carbonic anhydrase II and vacuolar H(+)-ATPase in osteoclasts; carbonic anhydrase II produces protons and vacuolar H(+)-ATPase transfers them into the lacuna. In acidified lacuna, cathepsin-K and matrix metalloproteinase-9 (MMP-9) are released from osteoclasts to degrade calcified tissues (Fuller et al. 2007).



c-Fms, M-CSF receptor; CTR, calcitonin receptor; MMP, matrix metalloproteinase; OSCAR, osteoclast-associated receptor; RANK, receptor activator of NF  $\kappa$  B; TRAF, TNF receptor-associated factor; TRAP, tartrate-resistant acid phosphatase.

Fig. 1. Schematic structure of osteoclast

Osteoclasts express unique cell adhesion structures called podosomes, which contain actin filaments. Podosomes are organized differently depending on the activity of the osteoclast; in bone-resorbing osteoclasts, podosomes form the actin ring, representing a gasket-like

structure, necessary for bone resorption, and in motile osteoclasts, podosomes are organized into lamellipodia (Latin lamella, a thin leaf; Greek pous, foot), the structure responsible for cell movement. Thus, the presence of actin rings and lamellipodia is mutually exclusive (Sarrazin et al. 2004). In 2004, Sarrazin et al. showed, using mature human osteoclasts extracted from the femurs and tibias of human fetuses, that osteoclasts have two subtypes of EP receptors, prostaglandin E<sub>2</sub> (PGE<sub>2</sub>), EP<sub>3</sub> and EP<sub>4</sub>, that mediate different actions of PGE<sub>2</sub> on these cells; activation of EP<sub>4</sub> receptors inhibits actin ring formation and activation of EP<sub>3</sub> receptors increases the number of lamellipodia (Sarrazin et al. 2004). Thus, PGE<sub>2</sub> directly inhibits bone resorption by human osteoclasts.

The cooperation of osteoclasts and osteoblasts is critical to maintain skeletal integrity in normal bones. After bone resorption by osteoclasts on normal bone tissues, osteoblasts subsequently rebuild bone in the lacunae resorbed by osteoclasts; this mechanism is called 'bone remodeling'. When the activity or number of osteoclasts is elevated compared with osteoblasts, the bone becomes fragile, that is, 'osteoporotic'. In addition, bone remodeling is disrupted in all bone diseases associated with changes in bone mass. Thus, bone remodeling is essential to retain both the structure and strength of normal bone.

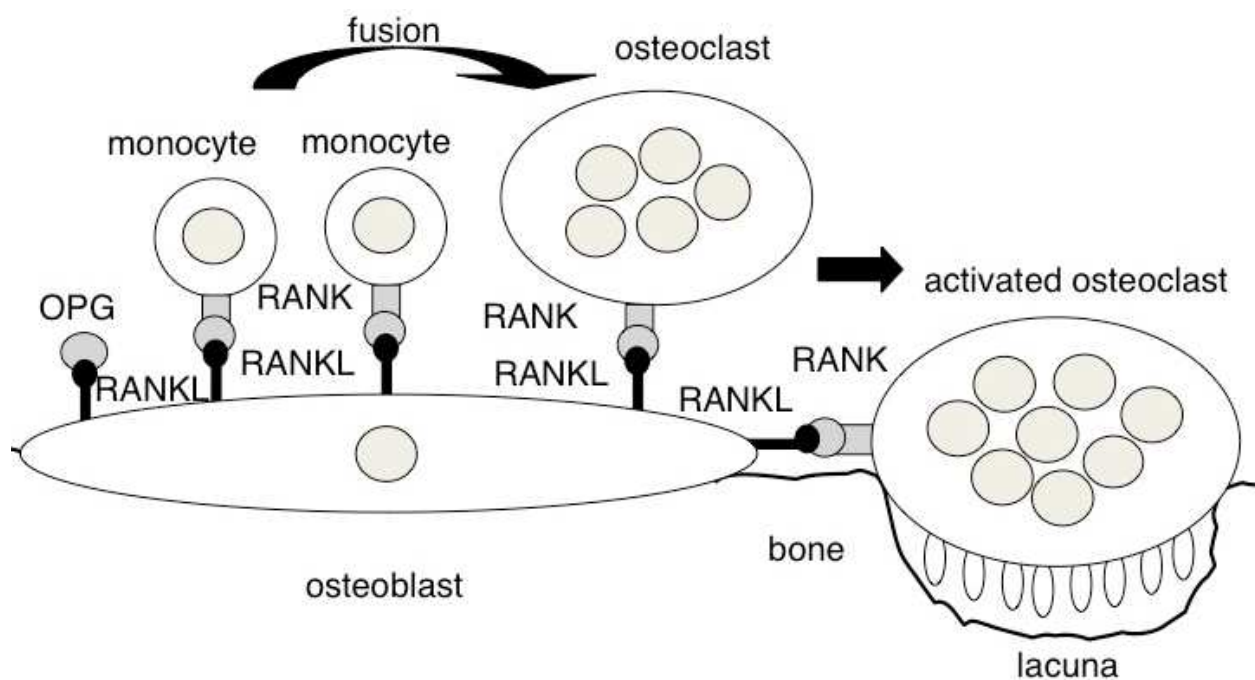
## 2.2 Origin of osteoclasts

The origin of osteoclasts was unclear until the late 1980s. In 1988, Takahashi et al. established a co-culture system using mouse spleen cells and osteoblasts to induce osteoclastogenesis *in vitro*, demonstrating that the origin of osteoclasts is hematopoietic cells and that osteoblastic cells are required for the differentiation of osteoclast progenitors in splenic tissues into multinucleated osteoclasts (Takahashi et al. 1988b). The precursor of osteoclasts was then revealed to be colony-forming unit-macrophage (CFU-M) or CFU-granulocyte/macrophage (CFU-GM) in bone marrow or spleen in mice. In 1990, Udagawa et al. demonstrated that osteoclasts are formed from murine macrophages (Udagawa et al. 1990). From these findings, Suda et al. hypothesized that bone marrow hemopoietic cells differentiate into osteoclasts through the stimulation of 'osteoclast-differentiation factor (ODF)' expressed on osteoblasts (Suda et al. 1995).

Finally, ODF, now termed RANKL, which induces osteoclastogenesis from monocytes or macrophages, was independently cloned by three groups in 1997 (Fig. 2) (Udagawa et al. 2002). RANKL is a member of the TNF superfamily of cytokines. The protein constructs a trimeric complex to bind its receptor, receptor activator NF- $\kappa$ B (RANK) (Lam et al. 2001). A decoy receptor is also cloned, which is designated as 'osteoprotegerin (OPG)' (Udagawa et al. 2002). In 2000–2001, we and other groups showed that T cells expressing RANKL induce osteoclastogenesis (Kong et al. 1999; Horwood et al. 1999; Kotake et al. 2001); in particular, we demonstrated osteoclastogenesis using human cells (Kotake et al. 2001), whereas others used murine cells (Kong et al. 1999, Horwood et al. 1999). In addition, in 2009, we reported that, in human osteoclastogenesis induced by RANKL, T-cell leukemia translocation-associated gene (TCTA) protein is required for cellular fusion (Kotake et al. 2009b).

## 2.3 Development of culture systems to form osteoclasts *in vitro*

Culture systems were developed to form osteoclasts *in vitro* in 1981–1988. In 1981, Testa et al. first succeeded in forming osteoclast-like multinucleated cells from feline marrow cells in long-term Dexter cultures (Testa et al. 1981). In 1984, using this feline marrow culture system, Ibbotson et al. showed that the formation of osteoclast-like cells is greatly stimulated



OPG, osteoprotegerin; RANK, receptor activator of NF  $\kappa$  B, RANKL, RANK ligand.

Fig. 2. Differentiation and activation of osteoclasts. A RANK-RANKL system induces both osteoclastogenesis from monocytes and the activation of mature osteoclasts.

by osteotropic hormones, such as  $1,25(\text{OH})_2\text{D}_3$ , PTH, and  $\text{PGE}_2$  (Ibbotson et al. 1984). In 1987, MacDonald et al. reported the formation of multinucleated cells that respond to osteotropic hormones in long-term human bone marrow cultures (MacDonald et al. 1987). In 1988, Takahashi et al. and in 1989, Hattersley et al. used marrow cells of mice to examine osteoclast-like cell formation from their progenitor cells (Takahashi et al. 1988a, Hattersley et al. 1989). Moreover, in 1988, Takahashi et al. established an innovative co-culture system using mouse spleen cells and osteoblasts to induce osteoclastogenesis *in vitro* (Takahashi et al. 1988b). Thus, since 1981, studies using osteoclastogenesis *in vitro* have been developed, and  $\text{PGE}_2$  was shown to up-regulate murine osteoclastogenesis using the marrow culture system *in vitro*.

#### 2.4 Role of osteoclasts in the pathogenesis of RA

Osteoclasts also play an important role in the pathogenesis of rheumatoid arthritis (RA). Since 1984, it has been reported that in bone destruction of RA, many activated osteoclasts are detected on the surface of eroded bone in the interface with synovial tissues (Shimizu et al. 1985). In addition, we have demonstrated that osteoclasts are detected in synovial tissues as well as eroded bone from patients with RA (Kotake et al. 1996). We have also reported that the number of precursor cells of osteoclasts increases in bone marrow adjacent to joints with arthritis (Kotake et al. 1992). Moreover, the amount of cytokines that induces osteoclastogenesis, such as IL-1, TNF- $\alpha$  and IL-6, is elevated in synovial tissues of patients with RA, while the amount of cytokines that induces osteoclastogenesis, such as IL-4 and IL-10, is decreased (Kotake et al. 1992; Kotake et al. 1996b; Kotake et al. 1997). Thus, patients with RA are likely to suffer from joint destruction as well as systemic osteoporosis, in which the number of osteoclasts increases, suggesting that osteoclasts play a critical role in the pathogenesis of RA.



## 2.5 Geranylgeranylacetone Inhibits the formation and function of human osteoclasts

The anti-ulcer drug geranylgeranylacetone (GGA), known as teprenon, is frequently used with nonsteroidal anti-inflammatory drugs (NSAIDs) in Japan. In 2005, we demonstrated that GGA inhibits the formation and function of human osteoclasts and prevents bone loss in tail-suspended rats and ovariectomized rats (Nanke et al. 2005). Vitamin K is also used to protect against osteoporosis. It has been reported that the inhibitory effect of vitamin K2 (menatetrenone) on bone resorption may be related to its side chain. GGA has almost the same chemical structure as the side chain of menatetrenone.

We hypothesized that GGA also has an inhibitory effect on osteoclastogenesis both *in vitro* and *in vivo*. GGA in pharmacological concentrations directly inhibited osteoclastogenesis from human monocytes induced by soluble RANKL. In addition, GGA induced the degradation of actin rings in mature osteoclasts, which was reversed by adding geranylgeranylpyrophosphatase. Moreover, GGA increased the bone mineral density of the total femur, proximal metaphysis, and diaphysis of femur in ovariectomized rats. GGA also prevented bone loss induced by hindlimb unloading in tail-suspended rats. These results indicate that GGA prevents bone loss by maintaining a positive balance of bone turnover through suppression of both the formation and activity of osteoclasts. In addition, in 2009, we also reported that GGA induces cell death in fibroblast-like synoviocytes from patients with RA (Nanke et al. 2009). Thus, GGA could be used to prevent and improve osteoporosis, especially in patients with RA.

## 3. 'Human osteoclastology' - Difference between human and mouse in immunology and cell biology

In basic science, murine cells are usually used, because these cells and experimental tools for them can be easily obtained. In addition, it is possible to create transgenic and knockout murine models. Indeed, biological science has made marked progress using murine cells and disease models. On the other hand, there are many disadvantages in studies using human cells, e.g., it is difficult to obtain human cells. In addition, it is impossible to perform an *in vivo* study.

However, to investigate the pathogenesis of RA, it is critical to investigate osteoclastogenesis using human cells. In bone cell biology, some cytokines show different functions between humans and mice. For example, M-CSF induces colony formation in mouse cells. In human cells, however, M-CSF usually induces the differentiation of progenitor cells of monocytes rather than colony formation, although Motoyoshi et al. initially reported that M-CSF induces colony formation in human macrophages in 1982 (Motoyoshi et al. 1982). On the other hand, some cytokines show different expressions between humans and mice; Kanamaru et al. reported that the expression of membrane-bound RANKL is limited in human T cells compared with mouse T cells (Kanamaru 2004). Moreover, the mouse CD4<sup>+</sup>CD25<sup>+</sup> regulatory subset can be isolated from all CD25<sup>+</sup> T cells regardless of their level of CD25 expression; however, when similar criteria are followed to isolate these cells from human blood, CD25<sup>+</sup> cells (high and low together) do not exhibit an anergic phenotype or significant suppressive function. In 2001, Baecher-Allan et al. demonstrated that, in humans, CD4<sup>+</sup>CD25<sup>high</sup> exhibit all the properties of regulatory T cells (Baecher-Allan et al. 2001). We also measured the percentages of CD4<sup>+</sup>CD25<sup>high</sup> T cells as regulatory T cells in patients with Behcet's disease (Nanke et al. 2008). In addition, Amadi-Obi et al. reported that a major immunological difference between humans and mice is the presence of CD4<sup>+</sup>T cells

producing IL-17, Th17 cells, in the peripheral blood of healthy humans, but not mice (Amadi-Obi et al. 2007). Recently, several studies have also demonstrated the existence of substantial numbers of human CD4<sup>+</sup> T cells that are able to produce both IL-17 and IFN- $\gamma$ ; the term “Th17/Th1” cells has been proposed (Annunziato et al. 2009).

In addition, two groups have reported the effects of PGE<sub>2</sub> on human osteoclastogenesis from monocytes alone stimulated by RANKL in the absence of osteoblasts, contrary to many reports on the stimulatory effects of PGE<sub>2</sub> on murine cells. In 1999, Itonaga et al. reported that PGE<sub>2</sub> inhibits osteoclast formation induced by RANKL in human peripheral blood mononuclear cell cultures (Itonaga et al. 1999). In 2005, Take et al. demonstrated that, unlike mouse macrophage cultures, PGE<sub>2</sub> strongly inhibits RANKL-induced osteoclast formation in human CD14<sup>+</sup> cell cultures (Take et al. 2005). In addition, they showed that human osteoclast progenitors produce a soluble unidentified factor(s) in response to PGE<sub>2</sub> that strongly inhibits RANKL-induced osteoclast formation not only in human CD14<sup>+</sup> cell cultures but also in mouse macrophage cultures. They tried to identify the soluble factors, and concluded that CD14<sup>+</sup> cells produce an inhibitor(s) that does not correspond to known inhibitory factors, such as GM-CSF, IFN- $\gamma$ , and IL-4. Thus, these reports demonstrated the possibility that PGE<sub>2</sub> differently plays a direct role in osteoclastogenesis from monocytes alone in the absence of osteoblastic cells between humans and mice.

Thus, in the study of human diseases, it is essential to investigate human osteoclastogenesis using human cells; the differences in species used in studies are critical to discuss the function of cytokines. We suggest that the term ‘human osteoclastology’ be used to describe studies on human osteoclastogenesis (Kotake 2009a, Kotake 2010).

#### 4. T-cell leukemia translocation-associated gene (TCTA)

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 (Aplan et al. 1995). TCTA mRNA is expressed ubiquitously in normal tissues, with the highest levels of expression in the kidney. TCTA has been conserved throughout evolution in organisms ranging from *Drosophila* to humans. A short open reading frame encodes a protein of 103 amino acid residues, Mr 11,300, without strong homology to any previously reported proteins. Of note, genomic Southern blots demonstrated a reduced TCTA signal in three of four small cell lung cancer cell lines, suggesting the loss of one of the two copies of the gene (Aplan et al. 1995). On the other hand, in 2005, it has been reported that TCTA interacts with SMA- and MAD- related protein 4 (SMAD4) in a proteome-scale map of the human protein-protein interaction network (Supplementary Table S2, line 6175) (Rual et al. 2005); however, the function of TCTA has not been clarified.

### 5. Role of TCTA protein in human osteoclastogenesis

#### 5.1 Hypothesis

Synovial tissues of patients with RA include factors regulating bone resorption by expressing cytokines such as RANKL, TNF $\alpha$ , IL-6, IL-17, and IFN $\gamma$  as described in Introduction. In addition to these cytokines, however, other factors expressed in synovial tissues may play a role in resorbing bone. We hypothesized that a novel factor in synovial tissues of patients with RA regulates osteoclastogenesis. To test this hypothesis, we tried

to identify novel peptides or proteins expressed in synovial tissues of patients with RA that regulate human osteoclastogenesis since 1996. We purified proteins from synovial tissues of patients with RA, using gel filtration chromatography, reverse-aspect HPLC, and mass spectrometry. We finally demonstrated that a peptide derived from the extracellular domain of TCTA protein inhibited both RANKL-induced human osteoclastogenesis and pit formation of mature human osteoclasts (Kotake et al. 2009b). We would like to present our findings as follows.

### 5.2 Purification of proteins from synovial tissues of patients with RA

Sixty-five grams of synovial tissues were obtained from 5 patients with RA at total knee replacement. The crude extract was obtained by homogenization of the synovial tissues. NH<sub>3</sub>Ac buffer was added to the freeze-dried crude extract. The supernatant was then applied to the column equilibrated with NH<sub>3</sub>Ac buffer and divided into two fractions, low molecular weight (MW) and high MW by first gel filtration chromatography. The low MW fraction was further applied to the column equilibrated with PBS by the second gel filtration chromatography. Proteins were eluted and protein concentration (A<sub>280</sub>) was monitored by UV absorption. Each fraction was added to the culture for human osteoclastogenesis to evaluate the activity on the osteoclastogenesis. Two fractions showed the inhibitory activity of osteoclastogenesis. These fractions were then subjected to ion-exchange chromatography. The “flow through” from the column showed the inhibitory activity on the osteoclastogenesis. The “flow through” was then applied to second gel filtration chromatography. Ten fractions from gel filtration chromatography showed the inhibitory activity on osteoclastogenesis. Thus, these fractions were applied to reverse-aspect HPLC. Thirty fractions were obtained by the reverse-aspect HPLC.

After each fraction obtained by reverse-aspect HPLC was concentrated, amino acid sequences were determined using a protein sequencer. Amino acid sequences (3-5 mers) were determined in 8 fractions. In addition, we tried to determine the sequence of each fraction by mass spectrometry; however, the sequences were not determined, although the total molecular weight of each peptide was speculated to be less than 1000 Da. Thus, we synthesized 8 peptides according to the sequences determined using the protein sequencer and evaluated the effect of each peptide on human osteoclastogenesis in vitro.

### 5.3 A small peptide including GQN inhibits human osteoclastogenesis

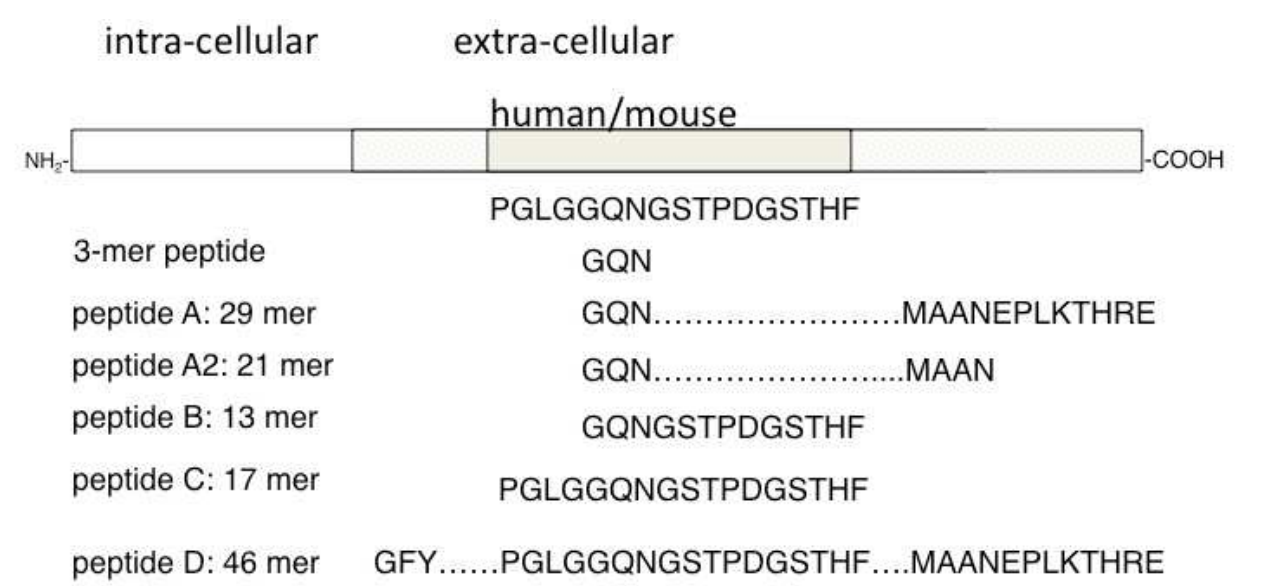
We finally revealed that the amino acid sequence of **Glycine-Glutamine-Asparagine (Gly-Gln-Asn; GQN)** alone in 8 synthesized peptides showed inhibitory activity in human osteoclastogenesis; **GQN** dose-dependently inhibited human osteoclastogenesis from peripheral monocytes by RANKL (IC<sub>50</sub>: around 30  $\mu$ M)(Kotake et al. 2009b).

When searching for proteins that include **GQN** using FASTA search, we found only 2 proteins in human proteins, CDW52 antigen (CAMPATH-1 antigen) and TCTA. In the FASTA search, it was possible to identify 3 mer peptides, but we did not find any other human proteins. Using “PeptideCutter”, by which it is possible to predict the site cleaved by enzymes in peptides, we synthesized 4 peptides from a sequence of CDW52 antigen and one peptide from a sequence of TCTA protein, which included **GQN**. A peptide from TCTA protein showed inhibitory activity on human osteoclastogenesis, but not peptides from CDW52 antigens.



**5.4 Peptide A from TCTA strongly inhibits human, but not mouse, osteoclastogenesis**

We then synthesized another 2 peptides, **GQNGSTPDGSTHFPSWEMAANEPLKTHRE** and **GQNGSTPDGSTHFPSWEMAAN**, using ‘PeptideCutter’, shown as “peptide A” (MW 3182.4) and “peptide A2”, respectively, from a sequence of TCTA gene protein (Fig. 3). Osteoclastogenesis was more potently inhibited by these peptides than by **GQN** or **GQNGST**. Peptide A most strongly inhibited osteoclastogenesis; IC50 level was around 1.6 μM. Osteoclastogenesis induced by soluble RANKL (sRANKL) was not inhibited by adding 1.6 μM of a scrambled peptide, SPFTGTKGSWNETAHPDHGNEERQAPMSL (MW, 3182.4), randomly designed from the sequence of peptide A. We finally synthesized 3 more peptides from a sequence of TCTA protein, also using “PeptideCutter”: “peptide B”: **GQNGSTPDGSTHF**, “peptide C”: **PGLGGQNGSTPDGSTHF**, and “peptide D”: **GFYGNTVTGLYHRPGLGGQNGSTPDGSTHFPSWEMAANEPLKTHRE**, which is the whole of the human extra-cellular domain (Fig. 3). Sequences of both peptide B and peptide C are included in a human/mouse identical sequence of TCTA protein (Fig. 3).



Structure of TCTA showing both intra-cellular (white bar) and extra-cellular (meshed bar) domains. Gray bar shows the human/mouse identical sequence in the extra-cellular domain.

Fig. 3. Structure of TCTA and sequences of peptides

Both peptide B and peptide C showed a weak inhibitory effect on human osteoclastogenesis from human peripheral monocytes. Peptide D did not show inhibitory activity. On the other hand, we also examined the effects of these peptides on mouse osteoclastogenesis; however, in contrast to our expectation, peptides B and C, which are included in the mouse sequence, did not inhibit ddY mouse osteoclastogenesis from mouse bone marrow cells stimulated by sRANKL and M-CSF. Peptides A and D did not show the inhibitory effects on mouse cells as we expected. Peptide A did not show cytotoxicity on human monocytes or cell proliferation on human monocytes (Kotake et al. 2009b).

**5.5 Peptide A failed to inhibit the expression of protein and mRNA of NFATc1, and mRNA of cathepsin K**

We then examined the effect of peptide A on the expression of both protein and mRNA of NFATc1, a key regulator of osteoclastogenesis under the signals of RANK-RANKL, in

osteoclasts. Peptide A appeared to weakly inhibit the expression of protein of NFATc1 in osteoclasts in immunohistological staining for NFATc1. Peptide A (0.8 – 3.2  $\mu$ M) also weakly, but not significantly, inhibited the expression of mRNA of NFATc1 in osteoclasts. In addition, peptide A did not inhibit the expression of mRNA of cathepsin K using the scrambled peptide as a control (Kotake et al. 2009b).

### **5.6 Peptide A inhibits pit formation of mature human osteoclasts in the culture of 3 days, but not 24 h**

We then examined the effect of peptide A on pit formation of mature human osteoclasts on Osteologic<sup>R</sup>. Mature osteoclasts formed from monocytes in culture with RANKL and M-CSF for 14 days were removed from plates. The mature osteoclasts then cultured on Osteologic<sup>R</sup> discs with peptide A or a scrambled peptide in the presence of RANKL and M-CSF for 24 h or 3 days. Peptide A as well as a scrambled peptide did not inhibit pit formation for 24 h. However, peptide A significantly inhibited pit formation in the culture of 3 days, although a scrambled peptide did not inhibit pit formation at all. Thus, peptide A showed inhibitory activity on mature human osteoclasts in the culture of 3 days, but not 24 h (Kotake et al. 2009b).

### **5.7 Peptide A suppresses the formation of large osteoclasts in the culture of mature human osteoclasts**

We then investigated the effects of peptide A on mature osteoclasts cultured with sRANKL and M-CSF for 3 days. In the control well with sRANKL alone in the absence of peptide A, larger osteoclasts were formed than osteoclasts cultured with sRANKL and peptide A. Huge osteoclasts were detected in the control well alone, but not in the well cultured with peptide A. Osteoclasts in the well cultured with peptide A showed usual size. The number of huge osteoclasts in the control well cultured without peptide A was significantly higher than in the well cultured with peptide A. Thus, we hypothesized that TCTA plays a role in the fusion of monocytes/macrophages, preosteoclasts and osteoclasts (Kotake et al. 2009b).

### **5.8 Polyclonal antibodies against TCTA inhibit both human osteoclastogenesis from monocytes and fusion of mature osteoclasts**

We constructed indirect competitive enzyme-linked immunosorbent assay (ELISA) using 2 polyclonal antibodies against TCTA protein. Standard curves showed specificity and high affinity of the antibodies against TCTA protein. To investigate our hypothesis further, we cultured monocytes with M-CSF and sRANKL in the presence of the polyclonal antibody, #1 or #2, both of which significantly inhibited human osteoclastogenesis, reducing the size of osteoclasts. In addition, polyclonal antibody #2 inhibited the fusion of mature osteoclasts (Kotake et al. 2009b).

### **5.9 TCTA protein is immunohistologically detected on human osteoclasts and macrophages**

Using antibody #1, TCTA protein was immunohistologically detected on human osteoclasts induced by sRANKL and M-CSF. TCTA protein was detected in the central area of cells or in the peripheral area of cells. TCTA protein stained in the peripheral area of cells was observed in both a pre-osteoclast with 2 nuclei and an osteoclast with 4 nuclei, using fluorescent microscopy. TCTA protein also detected human macrophages cultured with M-CSF (Kotake et al. 2009b).

### 5.10 Other findings on Peptide A

Peptide A as well as a scrambled peptide failed to disrupt the structure of actin rings of mature osteoclasts in the culture of 24h and 3 days. The amount of TCTA mRNA was significantly lower in human osteoclasts induced by sRANKL and M-CSF than in human macrophages cultured with M-CSF alone. Peptide A or the scrambled peptide, did not reduce the amount of TCTA mRNA in osteoclasts. TCTA mRNA was detected in human osteoclasts, monocytes, fibroblast-like synoviocytes, T cells, and PBMC by RT-PCR. TCTA protein was immunohistologically detected in cultured fibroblast-like synoviocytes using polyclonal anti-TCTA antibodies #1. TCTA protein was also immunohistologically detected in synovial tissues using polyclonal anti-TCTA antibodies #1. TCTA protein-positive cells were detected in synovial lining cells, but not in lymphoid folliculi with many lymphocytes. TCTA protein was significantly detected in human monocytes by flow cytometry using polyclonal anti-TCTA antibody #1 (Kotake et al. 2009b).

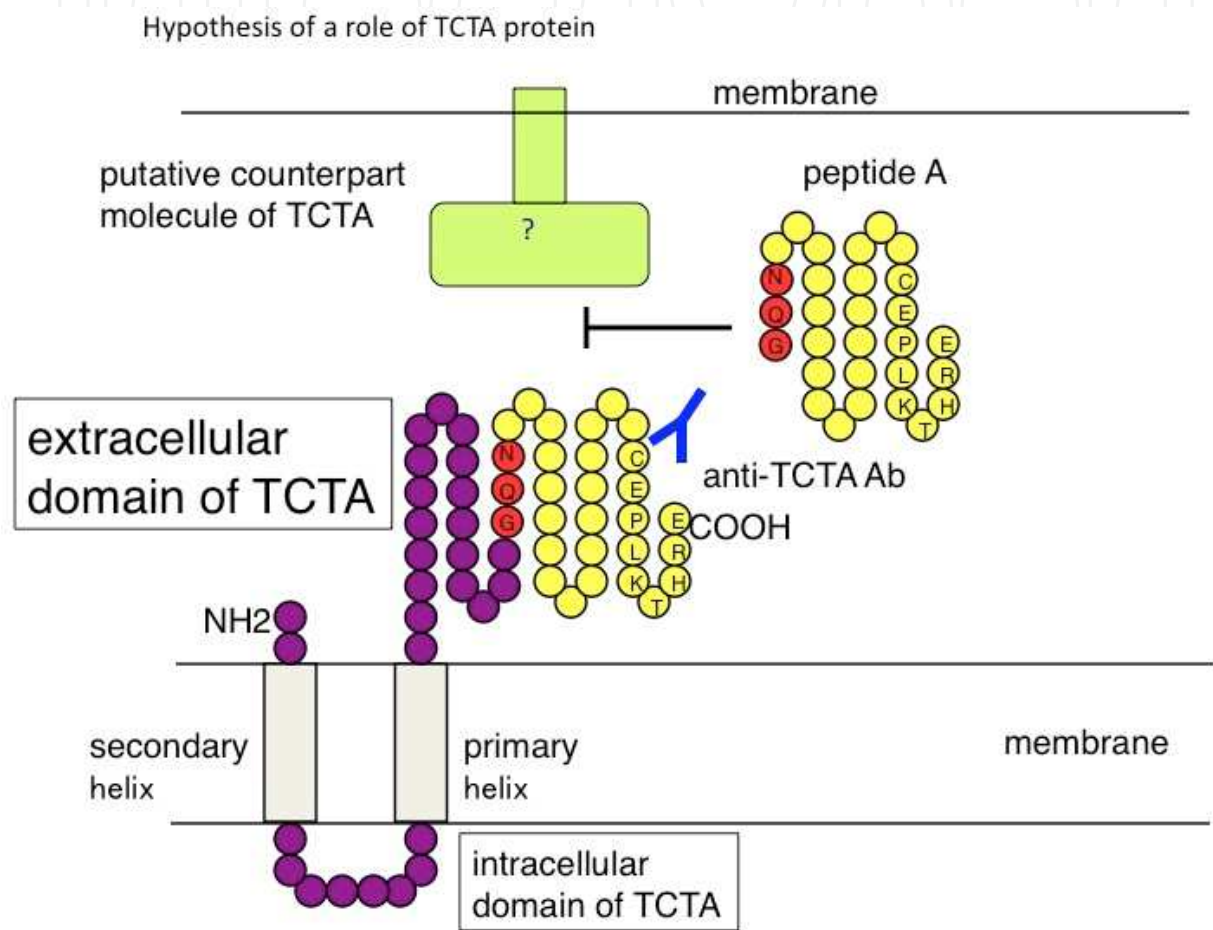
## 6. A novel hypothesis

We demonstrated that a novel peptide derived from the amino acid sequence of the extracellular domain of TCTA protein inhibited not only RANKL-induced human osteoclastogenesis from monocytes but also fusion of activated mature human osteoclasts (Kotake et al. 2009b). In the culture of mature osteoclasts, peptide A suppressed the formation of large mature osteoclasts after 3 days in the presence of sRANKL. In addition, although peptide A inhibited human osteoclastogenesis from monocytes with sRANKL, peptide A did not show significant inhibition of both protein and mRNA of NFATc1. The inhibitory effects of peptide A were not different among early, middle, and late phases of culture. In addition, the structure of actin rings of mature osteoclasts was not disrupted by peptide A. Thus, taken together, our findings suggest that the extracellular domain of TCTA may play a role in the fusion of osteoclast precursors and mature osteoclasts, and that peptide A may block the interaction of TCTA and a putative counterpart of TCTA (Fig. 4). Supporting our putative mechanism, Saginario et al. reported that, because the soluble extracellular domain of macrophage fusion receptor (MFR) prevents the fusion of macrophages *in vitro*, MFR belongs to the fusion machinery of macrophages (Sarginario et al. 1998).

Several studies have reported that peptides inhibit osteoclastogenesis (Choi et al. 2001; Ikeda et al. 2004; Jimi et al. 2004). Jimi et al. reported that, using a cell-permeable peptide inhibitor of the I- $\kappa$ B-kinase complex, the peptide inhibits RANKL-stimulated NF- $\kappa$ B activation and osteoclastogenesis both *in vitro* and *in vivo*. They also showed that this peptide significantly reduces the severity of collagen-induced arthritis in mice by reducing levels of TNF $\alpha$  and IL-1 $\beta$ , abrogating joint swelling and reducing the destruction of bone and cartilage (Jimi et al. 2004). They used 20  $\mu$ M of peptides to inhibit RANKL-induced mice osteoclastogenesis, whereas our peptide A inhibited RANKL-induced human osteoclastogenesis at 1.6  $\mu$ M of IC<sub>50</sub>. Thus, our peptide more effectively inhibits osteoclastogenesis, although the different efficacy of the peptides may be derived from the different species used: humans and mice.

The reasons why peptides B and C, included in the mouse sequence, did not inhibit the osteoclastogenesis from mouse bone marrow cells remain unclear. We used only 3 peptides, peptide B, peptide C, and mouse "peptide A", in the culture of mouse cells. The other peptides, including GQN, may inhibit mouse osteoclastogenesis. On the other hand, even if TCTA protein plays a role in fusion as discussed above, TCTA protein in mice may be less

important than the other molecules for fusion, such as, dendritic cell-specific transmembrane protein (DC-STAMP) (Kukita et al. 2004; Yagi et al. 2005), CD9 (Yi et al. 2006), CD47 (Lundberg et al. 2007; Yago et al. 2006), macrophage fusion protein (MFR)(Lundberg et al. 2007; Saginario et al. 1998; Yago et al. 2006), E-cadherin (Mbalaviele et al. 1995; Vignery 2000; Vignery 2005), meltrin- $\alpha$  (ADAM12) (Abe et al. 1999), or CD44 (Suzuki et al. 2002). These findings also underline the difference of differentiation of osteoclasts between human and mice, supporting the importance of the term, ‘Human osteoclastology’, as mentioned in section 3 in this article.



TCTA protein plays an important role in cellular fusion in human osteoclastogenesis from monocytes and mature osteoclasts. Both peptide A and antibodies block the interaction between TCTA protein and a putative counterpart of TCTA protein.

Fig. 4. Possible mechanism of human osteoclastogenesis by peptide A and antibodies against TCTA protein. (Structure of TCTA is derived from SOSUI; <http://sosui.proteome.bio.tuat.ac.jp>)

In conclusion, we demonstrated that peptide A and polyclonal antibody against TCTA protein inhibited not only human osteoclastogenesis from monocytes but also the further maturation of mature human osteoclasts *in vitro* (Kotake et al. 2009b). Our findings suggest that TCTA protein plays an important role in cellular fusion in human osteoclastogenesis from monocytes and mature osteoclasts. Thus, peptide A may show the same inhibitory function *in vivo*, offering an effective therapeutic approach for inhibiting bone resorption.



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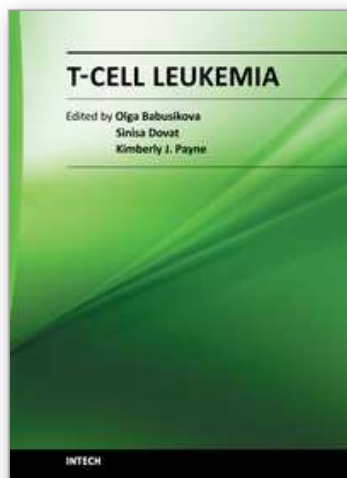
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The purpose of this book is to provide a comprehensive review of the scientific advances in T-cell malignancies and to highlight the most relevant findings that will help the reader understand both basic mechanisms of the disease and future directions that are likely to lead to novel therapies. In order to assure a thorough approach to these problems, contributors include basic scientists, translational researchers and clinicians who are experts in this field. Thus, the target audience for this book includes both basic scientists who will use this book as a review of the advances in our fundamental knowledge of the molecular mechanisms of T-cell malignancies, as well as clinicians who will use this book as a tool to understand rationales for the development of novel treatments for these diseases.

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