IDENTIFICATION AND CHARACTERIZATION OF THE INTERACTIVE PROTEINS WITH CYTOTOXIC T – LYMPHOCYTE ANTIGEN – 2α

(CTLA-2α と相互作用するタンパク質の同定とその性質の研究)

The United Graduate School of Veterinary Science

Yamaguchi University

Bui Thi To Nga

2014

ACKNOWLEGEMENTS

I would like to extend my gratitude to many people who helped to bring this research project to fruition.

In the first place**,** my greatest appreciation to my course supervisor, Prof. Dr. Yoshimi Yamamoto, who has always been awesome, creative, gung-ho and full of great ideas. His positive approach to life, passion for work and continuous sharing of knowledge is something that I shall never forget for life. To the love of my life and my dearest family members, thank him for always being there in rain and shine. I'd have never made it this far without his encouragement and constant support.

I am indeed thankful to Prof. Kusakabe, Prof. Nishigaki, Prof. Sato, Prof. Otoi, Prof. Yamano (Tottori University), Dr. Yamamoto (Faculty of Medicine) for their guidance, stimulating suggestions to my research.

I would like to thank my parents and two sisters. They were always supporting me and encouraging me with their best wishes.

Finally, I would like to thank to my family for their love, patience and encouragement. Especialy to my husband, Trinh Ngoc Ha, who always there cheering me up and stood by me through the good and bad time. I owe a special thanks to my lovely children, Ngoc Khanh & Minh Quang, for their patience and encouragement during my Ph.D study. This accomplishment would not have been possible without them. Thank you.

CONTENT

LIST OF THE FIGURES

HEK293T cells

Figure 15: Distribution of CTLA-2α and cathepsin L in the mouse placenta at various placentation periods.

Figure 16: Co-localization of cathepsin L, cathepsin C, and TINAGL1 with CTLA-2α in 8 and 10 dpc mouse placenta

LIST OF TABLES

General introduction

1. BACKGROUND

Cysteine proteases

Proteases (also termed as peptidase or proteinase) are the enzymes that play the central role in the degradation of protein by hydrolyzing peptide bond that joins amino acids together in proteins. Proteases are found in all the living things from viruses to mammalia. These enzymes are involved in a multitude of physiological progresses from simple digestion of food proteins (e.g., digestive proteases in saliva, stomach and intestines) to highly regulated cascades (e.g., the complement system and apoptosis pathways). Furthermore, they are associated with various disease such as arthritic joint erosion, atherosclerotic plaque formation, tumor invasion and metastasis thus represent excellent target for therapeutic intervention (Bromme and Wilson, 2011). Proteases can be categorized based on their substrate specificities or mechanisms of catalysis. Upon the basis of the mechanism of peptide hydrolysis, seven major protease classes are known: serine, cysteine, threonine, aspartic, glutamic, asparagine and metallo catalytic type. In more than 670 proteases expressed from human genome, 31% are serine proteases, 33% metallo proteases, 4% aspartic protease, and 25% cysteine protease (http//merops.sanger.ac.uk).

Proteases in which the thiol group of a cysteine residue serves as the nucleophile in catalysis are defined as cysteine protease (Barret and Woessner, 1998). Cysteine proteases are widely distributed in a variety of biological tissues and fluid, where they involved in the process of intra- and extra-cellular protein degradation and turn over (Reddy et al., 1995). Papain-like cysteine proteases, which are also known as cysteine cathepsins, is the largest subfamily among the cysteine protease class (clan CA, family C1). Papain-like cysteine proteases have

been increasingly recognized as critical enzyme activities in degenerative, invasive, and immune system related disorders.

TABLE 1

CYSTEINE PEPTIDASES BELONG TO C1 FAMILY

Peptidases and Homologous	**MEROPS ID**
papain	C01.001
chymopapain	C01.002
caricain	C01.003
cathepsin V	C01.009
cathepsin X	C01.013
cathepsin-1	C01.016
cathepsin F	C01.018
cathepsin M	C01.023
cathepsin L	C01.032
cathepsin L1 (*Fasciola* sp.)	C01.033
cathepsin S	C01.034
cathepsin O	C01.035
cathepsin K	C01.036
cathepsin W	C01.037
cathepsin P	C01.038
cathepsin Q	C01.039
cathepsin H	C01.040
tubulointerstitial nephritis antigen	C01.973
tubulointerstitial nephritis antigen-related protein	C01.975
cathepsin B	C01.060
dipeptidyl-peptidase I (cathepsin C)	C01.070

Source: http//merops.sanger.ac.uk

These proteases have obtained little attention since it was thought that their functions are restricted in lysosomes. Recent efforts in the understanding of the physiological roles of these enzymes using protease-deficient mouse models has changed this view. It becomes more and more clear that papain-like cysteine proteases fulfill specific functions in extracellular matrix turnover, antigen presentation, and processing events and that they may represent attractive target for the development of therapeutic inhibitors for major diseases such as osteoporosis, arthritis, immune-related diseases, atherosclerosis, cancer (Lecaille et al., 2002).

Inhibitors of cysteine cathepsin

The papain family is the largest family, the members of which include a wide range of enzymes from both prokaryotes and eukaryotes, encompassing bacteria, plants, vertebrates and invertebrates (Bertie and Storer, 1995). The proteinases of this family are implicated in a number of degradative, invasive and pathological processes. Therefore, cysteine proteinases of the papain family represent attractive targets for the development of therapeutic inhibitors because of their involvement in abnormal physiological processes. However, since they are involved in important roles in normal protein turnover process and also due to their broad substrate specificity, the development of highly selective inhibitors has been a great challenge (Wiederanders, 2003). Since the early 1970s, a variety of low molecular weight inhibitors of cysteine proteases have been discovered.

All cysteine cathepsins are composed of a signal peptide, a propeptide, and a catalytic domain (Fig.1). The signal peptide, which consists of 10-20 amino acids in length, is cleaved off at the site of the translocation into the endoplasmic reticulum. The propeptide is cleaved in the increasingly acidic environment of the

endosomal/lysosomal system resulting in a fully active catalytic domain of the cathepsins. To date, the pro-region has three known functions (Bromme and Wilson, 2011): 1) acts as a scaffold for protein folding of the mature region; 2) works as chaperon for the transport of proenzyme to the endosomal/lysosomal compartment; 3) potent and highly selective inhibitor of the cognate enzyme.

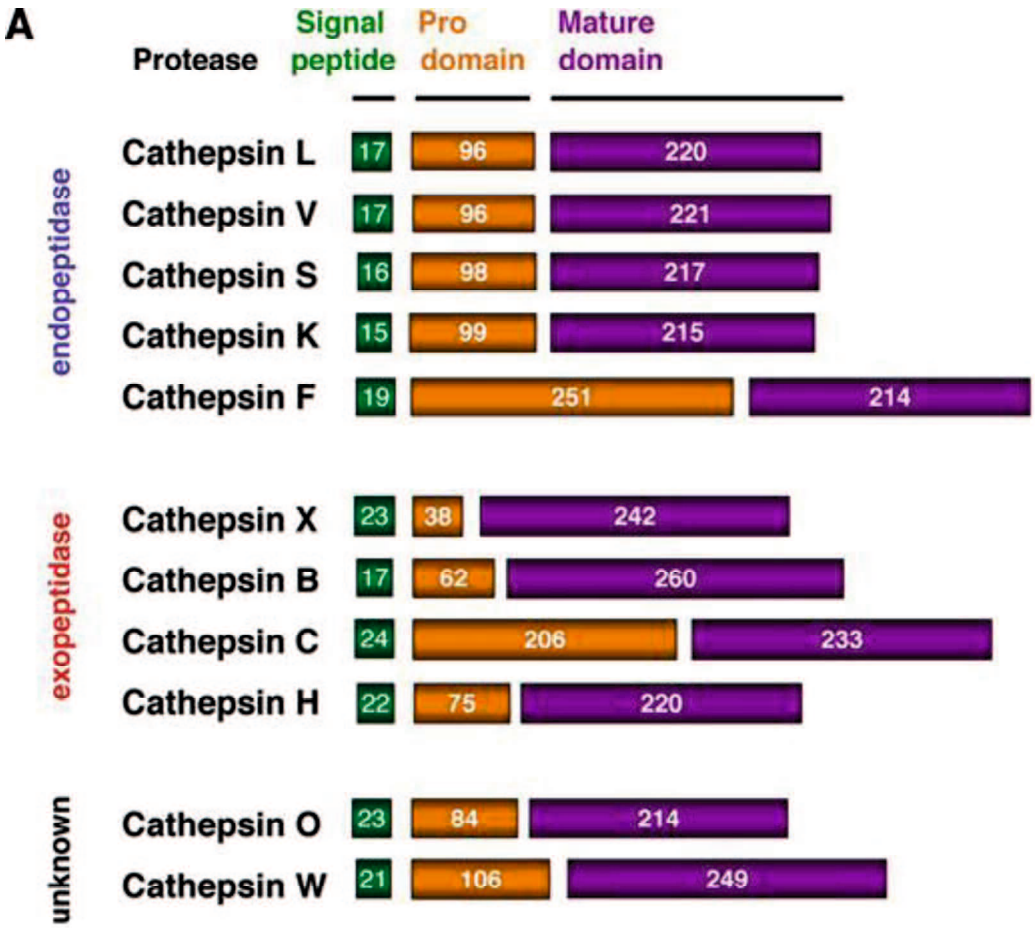


Figure 1: Schematic representation of the protease domains of human cathepsins *(Source: Bromme and Wilson, 2011)*

Cytotoxic T-lymphocyte antigen-2α

The expression of cytotoxic T-lymphocyte antigen-2 was originally detected in mouse activated T-cells and mast cells (Denizot et all., 1989). It was described as a transcript that was induced upon activation of T-lymphocytes. Two similar, but distinct

CTLA-2 transcripts were identified and named as CTLA-2α and CTLA-2β. CTLA-2αβ are described to have evolved from a cysteine proteinase ancestor gene through process of repeated duplication and translocation. CTLA-2α and CTLA-2β are highly homologous proteins and both of them contain 110 amino acid residues with an additional N-terminal hydrophobic amino acid sequence. The protein sequences of CTLA-2α and β are homologous to the proregion of mouse cathepsin L with the 42% homology for CTLA-2α and 36% homology for CTLA-2β (Yamamoto et al., 2002). Cysteine proteinases such as cathepsin L and cathepsin B are synthesized as inactive proenzymes (Turk B et al.; 2000). The N- terminal proregions of these proteases, which are removed during the activation process, act as potent inhibitors of mature enzymes (Turk B et al.; 2000). Since the proregion can fold independently, interest in novel inhibitor proteins from this region is increasing. The proregion is a part of the enzyme but CTLA-2α is expressed independently and has been designated as a member of the I29 peptidase inhibitor family (http://merops.sanger.ac.uk).

CTLA-2α has been characterized and expressed as a recombinant protein and has been shown to selectively inhibit mouse cathepsin L-like cysteine proteinases (Kurata et al., 2001). Previous studies suggested that CTLA-2β can inhibit cathepsin L-like cysteine protease, but is less selective than CTLA-2α (Delaria et al., 1994). One of the essential amino acid residues of CTLA-2α that is necessary for its inhibitory potency is cysteine 75 (C75), which is located in the vicinity of the C-terminal region and interacts with the active-site cleft of the enzyme (Deshapriya et al., 2010).

Previous studies showed that CTLA-2α was expressed in pregnant uteri of mice (Campo et al.; 1996) while CTLA-2β was expressed in early pregnant uteri,

suggesting that CTLA-2β is a regulator of embryo implantation (Cheon et al.; 2004). In the mouse brain, CTLA-2α mRNA was found to be preferentially localized within neuronal populations (Luziga et al., 2007). The CTLA-2α protein has also been detected in neuronal dendrites and axons (Luziga et al., 2008) suggesting that CTLA-2α is involved in memory establishment in mouse brain. Studies also show that CTLA-2α is involved in the formation of regional immunity in the eye (Mochizuki et al., 2010). Retinal pigment epithelium-derived CTLA-2α has the ability to generate T regulatory cells (Sugita et al., 2008, 2009, 2011).

Objectives of this doctoral thesis

Even though CTLA-2 is the most studied of the propeptide-like cysteine protease inhibitors, its inhibition mechanisms have not been fully determined. Many attempts have been made to explore more functionally specific sequences or residues for inhibition in the proregion of cysteine protease. The essential amino acid residues of CTLA-2α necessary for its inhibitory potency have been identified. It is suggested that a disulfide bonding between CTLA-2α and the enzyme has been established when the inhibitory reaction happened (Deshapriya et al., 2010).

On the other hand, in most of previous studies, CTLA-2α has been proposed to act as cathepsin L inhibitors on the above described physiological mechanisms. Its physiological role is not clear. A recent study revealed that CTLA-2α induced cAMP/PKA-promoted apoptosis in murine T-lymphoma cells and cardiac fibroblasts (Zhang et al., 2011). This inducing mechanism was not related to the ability of CTLA-2α to inhibit cathepsin L and indicated that CTLA-2α performs various physiological functions that have yet to be identified.

Armed with confidence based on essential information from previous studies, goal-oriented objectives were carried out step by step throughout my PhD studies to further elucidate the inhibitory mechanism and physiological functions of CTLA-2α

The results of all these objectives where discussed in further details in five chapters. In Chapter 1, the general information about cysteine protease and CTLA-2α is presented. Chapter 2 reports the yeast screening of the proteins interact with CTLA-2α. The highlights in Chapter 3 comprise of the association features of CTLA-2α and other proteins *in vitro* and *in vivo*, the novel explore of the CTLA-2α inhibitory activity toward cathepsin C in cells. The analysis and results of the co-localization of CTLA-

2α and its interactive proteins in cells and tissue were discussed in the Chapter 4. The final chapter, Chapter 5 was spent for discussion.

Study I

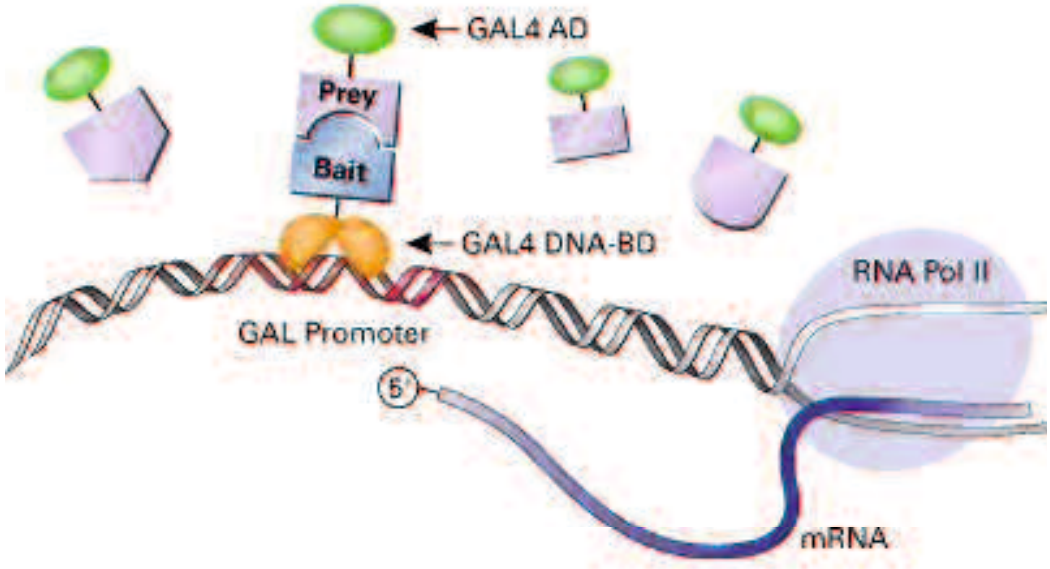
Interactions between CTLA-2α and cathepsin L, cathepsin C and TINAGL1

II.1. LITERATURE REVIEW

This chapter reports the novel finding about the interaction between CTLA-2α and others protein by yeast two-hybrid system. To date, cathepsin L has been known as the only target protein of CTLA-2α. Therefore, understanding about the functions of this protein had been restricted in the manner of one of potent inhibitor of cathepsin L. The report from Zhang et al.,(2011) has opened the door to new view about CTLA-2α. They have found that CTLA-2α expression is regulated by cAMP/protein kinase A- dependent mechanism and may contribute to cAMP/PKA-promoted cells death in cells. Cathepsin L has been known to be involved in immune system but the role in apoptosis is not clear if it is promote or anti – apoptotic. Interestingly, Zhang group has found that cAMP/PKA up-regulated cathepsin L activity but over-expression of CTLA-2α protein in cells does not change its activity. In simpler terms, the contribution of CTLA-2α to cAMP/PKA-promoted apoptosis does not related to the ability of CTLA-2α to inhibit cathepsin L. It was raised a question whether another protease participate in regulation of apoptosis by CTLA-2α or in other words CTLA-2α has others target protein apart from cathepsin L. It will thus be of interesting to identify the proteins association with CTLA-2α. To achieve the result, the yeast two-hybrid system has been applied.

The “two-hybrid system” developed by Fields and Song (1989) can be used to look for new interacting proteins that are likely to association according to genetic or biochemical data. This system is a molecular genetic based on the advantage of the flexible structure of the yeast transcription factor Gal4. The Gal4 protein contains two domains, DNA-binding domain (DNA – BD) and activation domain (AD). These domains need not to be part of the same protein to activate the transcription. When fused separately the two domains into two unrelated but interacting proteins, the

transcription will be activated as the result of the protein-protein interaction (Fig. 2). Usually, the search for new interacting proteins using yeast two-hybrid system has been carried out by co-transforming two vectors encoded bait protein cDNA and prey proteins cDNA into yeast strain.



Source: Clontech

Figure 2: The two – hybrid principle. Bait protein is expresses as a fusion to the Gal4 DNA – BD, while the libraries of prey protein are express as fusion to the Gal4 AD. When bait and prey fusion protein interact, the DNA – BD and AD are brought in to proximity to active transcription of four independent reporter genes.

In this study, CTLA-2α was used as a bait protein and the prey proteins were searched from the universal, embryonic, and brain cDNA libraries of mouse.

II.2. MATERIALS AND METHODS

Yeast two-hybrid screening

Yeast two-hybrid screenings were performed using the Matchmaker Gold Yeast Two-Hybrid System (Clontech. CA, USA). cDNA fragments encoding mouse CTLA-2α were cloned into the pGBKT7 vector containing the GAL4 DNA binding domain to generate the bait plasmid, pGBKT7.CTLA2α. This construct did not have toxic effects or autonomously activate the reporter genes in Y2HGold yeast strain. Universal, embryonic, and brain cDNA libraries from mouse were purchased (Mate & plate^{TM} library, Clontech), and constructed in the pGADT7 vector containing a GAL4 activation domain and transformed into yeast strain Y187. Positive clones were selected based on their ability to grow on synthetic dropout media minus leucine, tryptophan (SD/DDO) and minus leucine, tryptophan, histidine, adenine (SD/QDO) and exhibit X-α-Gal activity.

Clone isolation and interaction analysis

Yeast colony PCR analysis was performed to eliminate duplicates containing the same AD/library inserts. Matchmaker Insert Check PCR Mix 2 (Clontech) has been applied to amplify the prey library inserts. The present of more than a single band in electrophoresis gel indicated the present of more than one prey plasmid in a yeast cell. Such inserts will be re-selected and purified by precipitation PCR product. Next, streaking on DDO/X media has been done 2 times to segregate the non-interacting preys. The only blue colonies have been selected. Plasmid DNAs were isolated from the positive clones (Easy Yeast Plasmid Isolation Kit, Clontech) and transformed into *Escherichia coli* JM109. Positive prey clone-derived plasmid DNAs

were rescued by ampicillin resistance and positive cDNA were identified by nucleotide sequence analysis with the BLAST database (www.ncbi.nlm.nih.gov/blast). Interactions were verified by co-transforming the pGADT7 vector containing the identified cDNA with the bait pGBKT7 CTLA2α into the yeast AH109 strain followed by selection on SD/DDO/X and SD/-QDO/X media.

Expression construct, cell culture and transfection

cDNAs encode CTLA-2α, Cathepsin L, Cathepsin C and TINAGL1 were cloned into pAcGFP plasmid (for bait) and pProlabel plasmid (for preys) to generate pGFP-CTLA and pPro-CtsL, pPro-CtsC, pPro-TINAGL.

HEK293T (Human Embryonic Kidney) cells were cultured in DMEM (Wako) supplemented with 10% fetal bovine serum at 37 C in a humidified atmosphere containing 5% CO2. DNA transfection of HEK293 cells was carried out at 60% – 80% confluent using ScreenFect A (Wako)

Co-immunoprecitaion assay for cells

The Matchmaker Chemiluminescent Co-IP System was performed to confirm interactions between CTLA-2α and Cathepsin L, Cathepsin C and TINAGL1 which have been identified through the yeast two-hybrid screening. This is a co-immunoprecipitation method that utilizes a fluorescent AcGFP1 tag and the enzymatic ProLabel reporter for chemiluminescent detection of physical interactions between proteins that are expressed and posttranslationally modified in mammalian cells. After the AcGFP1- tagged bait and the ProLabel-tagged prey fusion proteins are co-expressed in the cells, the Matchmaker Chemiluminescent Co-IP Kit is

performed to immunoprecipitate the bait. Then, the co-immunoprecipitation complexes are quantified by assaying for Prolabel activity using Prolabel Detection Kit.

HEK293T cells were cultured on the 75cm2 flask and co-transfected with pGFP-CTLA and pPro-CtsL, pGFP-CTLA and pPro-CtsC, pGFP-CTLA and pPro-TINAGL. The cells co-transfected with pGFP-lam and pPro-T were used as negative control, pGFP-p53 and pPro-T were positive control. After 48h, cells were washed twice with PBS and trypsinized. The cells pellets then were collected and further lysated according to the manufacturer's instructions. The lysated samples were subjected to the co-immunopecipitation assay using Protein G Plus/A Agarose Beads and anti-AcGFP Ab. The samples were incubated with 1 µl of anti-AcGFP Ab in 2h on a rotator at 4^0C followed by an overnight incubation with washed agarose beads. Next morning, the beads were pelleted at 9000 rpm/10 secs. After washing with the Wash Buffer 1, the pellets were suspended in the Wash Buffer 2 and ready for the Prolabel activity detection. The Prolabel Detection Kit was applied to assay the prey expressions. The Wash Buffer 2 was removed and the samples with beads were resuspend in 80 µl of the lysis/complementation then transferred to a well in the 94-well plate. To each well, add 30 µl of subtrate mix and read the Prolabel activity from the samples at 0, 20, 45 and 70 min after addition of the subtrate. The results were sumarized after analyzing the plot the Prolabel reading.

Generation of antibodies

Recombinant CTLA-2α and cathepsin L were purified using methods described previously with minor modifications (Kurata et al., 2003). Affinity purified

rabbit anti-CTLA-2, anti-cathepsin L IgGs and chicken anti - anti-CTLA-2, anti-cathepsin L IgYs were obtained as described previously (Takahashi et al., 1992; Camenisch et al., 1999; Luziga et al., 2008). In brief, egg yolk immunoglobulin fractions were prepared from eggs laid by hens immunized against recombinant CTLA-2α and cathepsin L. Chicken anti-CTLA-2α and anti-cathepsin L IgYs were affinity purified through columns with recombinant protein conjugated resins. Mouse (sc-74590) and rabbit (sc-13986) anti-cathepsin C antibodies were purchased from Santa Cruz Biotechnology, Inc (TX, USA). An anti-TINAGL1 antibody (ab107679) was purchased from Abcam (Cambridge, MA, USA).

Co-immunoprecipitation (Co-IP) for tissue

Complexes of CTLA-2α/cathepsin L and CTLA-2α/cathepsin C were isolated from pregnant mouse uterus tissue. All experiments using mouse uteri conformed to the 1973 law concerning the protection and control of animals (guidelines for animal experimentation) in Japan. Mice were anesthetized by diethyl ether, and uteri were taken out and quickly rinsed in 0.9% sodium chloride, then frozen, and stored at -80ºC until future use. Frozen uteri were thawed, cut into pieces, and homogenized in 4 vol. of buffer (0.1M Tris-Cl at pH 7.4 containing 0.3 M NaCl, 1 mM EDTA, and 0.1% Triton X-100). Insoluble materials were removed by centrifugation (13.000 rpm, 5 min) and the supernatant was incubated at 37ºC for 10 min. In order to isolate the complexes of CTLA-2α/cathepsin L and CTLA-2α/cathepsin C, the incubated sample was applied to anti – CTLA-2α IgG coupled beads (Dynabeads Co-immunoprecipitation Kit, Invitrogen, Oslo, Norway). The unbound proteins were removed by washing with PBS and the target proteins were specifically eluted according to the manufacturer's instructions. Control samples were co –

immunoprecipitated with the beads omitting anti - CTLA-2α IgG. Immunoblotting analyses were performed as previously described (Luziga et al., 2008).

Pull-down assay

The molecular weight of TINAGL1 is close to that of IgG. Therefore, It is impossible to distingue between TINAGL1 and IgG leaking from the beads of the Co-IP assay. A pull-down assay was performed to determine the complex of CTLA-2α/TINAGL1. Human Embryonic Kidney (HEK293T) cells were cultured in DMEM (Wako, Osaka, Japan) supplemented with 10% fetal bovine serum at 37^0C in a humidified atmosphere containing 5% CO_2. Full-length cDNA encoding TINAGL1 was cloned into a pFUΔss plasmid (modified from pFUSE-hIgG2-Fc1, InvivoGen, CA, USA) to generate pFU.TINAGL1 was transfected into HEK293T cells at 60% – 80% confluence using ScreenFect A (Wako). The recombinant CTLA-2α protein, which contained a His-tag sequence, was prepared as described above. Recombinant CTLA-2α protein (7μg/ml) was added into the culture medium 24 h after transfection. The culture medium was then collected 7 h later and subjected to the pull-down assay using His–tag resin (Dynabeads TALON, Invitrogen). The proteins recovered were subjected to 12% SDS-PAGE and analyzed by immunoblotting. For negative control, PBS was used in place of CTLA-2α.

3. RESULTS

3.1. CTLA-2α interacts with cathepsin L, cathepsin C and TINAGL1 in yeast

The yeast two–hybrid system with the bait plasmid pGBKT7.CTLA2α was used to screen cDNA libraries derived from mouse tissues in order to identify mouse proteins that interact with CTLA-2α. A total 8 x 10^7 diploid cells were screened yielding 91 positive clones, after eliminating the duplicates clones and segregation 17 of which demonstrated strongly growth on DDO/X media. The DNAs of these clones were isolated, sequenced, and aligned using the NCBI BLAST alignment system. As can be seen in the Table 2, two clones have not matched with any sequence in database (marked as non-sense: number 5 and 13), clone 18 has matched with unknown protein. The rest colonies have been selected for further step to confirmation the interaction.

Among the candidate proteins, cathepsin L, cathepsin C, and TINAGL1 were identified to interact with CTLA-2α. The growth of blue colonies on the SD/-QDO/X medium was observed in yeast cells co–transformed with pGBKT7.CTLA2α/ pGADT7.cathepsinL, pGBKT7.CTLA2α/pGADT7.cathepsinC and pGBKT7.CTLA2α/pGADT7.TINAGL1 (Fig. 3A).

The alignment of the cathepsin L, cathepsin C and TINAGL1 proteins was examined (Fig. 3B). The three proteins belong to the papain family, C1A (http://merops.sanger.ac.uk). Unlike cathepsin L and cathepsin C, TINAGL1 is not a peptidase. Its active cysteine residue was replaced by a serine residue (as highlighted in box in Fig. 3B), resulting in a proteolytically inactive protein.

The alignment of CTLA-2α and pro-region of cathepsin L (Fig. 3C), catepsin C (Fig 3D) proved the highly homology between CTLA-2α and the pro region of these

two cysteine proteases, 30% homology for the pro region of cathepsin L and 25% homology for pro region of cathepsin C.

3.2. CTLA-2α interacts with cathepsin L, cathepsin C and TINAGL1 in mammalian cells.

Co-immunoprecitaion assay was performed to confirm the interactions between CTLA-2α and Cathepsin L, Cathepsin C and TINAGL1 on the mammalian cells. The Figure 4 shows the result of Prolabel assay to detect the Prolabel activity from the complexes between CTLA-2α and each of the three proteins. The Prolabel activities were observed from the positive control and 3 samples with varying levels. The highest level was obtained from the positive control with 1195 RLU after 70 min incubation while no observed activity from negative control. The cells co-transfected with CTLA-2α and TINAGL1 showed the significant increasing in the level of the signal, reached 52 RLU after 45 min and 128 RLU after 70 min incubation with chemiluminescent substrate. Similarly trend but ten times lower signal was observed from the cells co-transfected with CTLA-2α and cathepsin L, CTLA-2α and cathepsin C: 14 RLU and 10 RLU after 70 min incubation, respectively. These results indicate that these three prey proteins have been co-immunoprecipitated with CTLA-2α by the Co-IP assay. In other word, CTLA-2α interacts with cathepsin L, cathepsin C and TINAGL1 on the mammalian cells.

Table 2 : Sequence analysis for positive candidates from the screenings

No	GeneBank Accession No.	Matched protein
1	BC017684	cytidine monophosphate (UMP-CMP) kinase 1
2	BC057195	ankyrin repeat domain 17
3	BC090389	ribosomal protein S20
4	BC027313	1600012H06Rik protein
5		non sense
6	BC003420	DnaJ (Hsp40) homolog, subfamily A, member 2
7	BC067054	regulation of nuclear pre-mRNA domain containing
8	AL672278	DNA sequence from clone RP23-465H23
9	NP446034	**Tubulointerstitial nephritis antigen-like protein 1**
10	BC067063	**cathepsin C**
11	BC067063	**cathepsin C**
12	J05287	lysosomal membrane glycoprotein (LAMP-2)
13		non sense
14	BC141031	splicing factor, arginine/serine-rich 18
15	BC034754	archain 1
16	U85089	thioredoxin
17	U85089	thioredoxin
18		un known
19	NP034114	**Cathepsin L**
20	BC085267	splicing factor, arginine/serine-rich 5

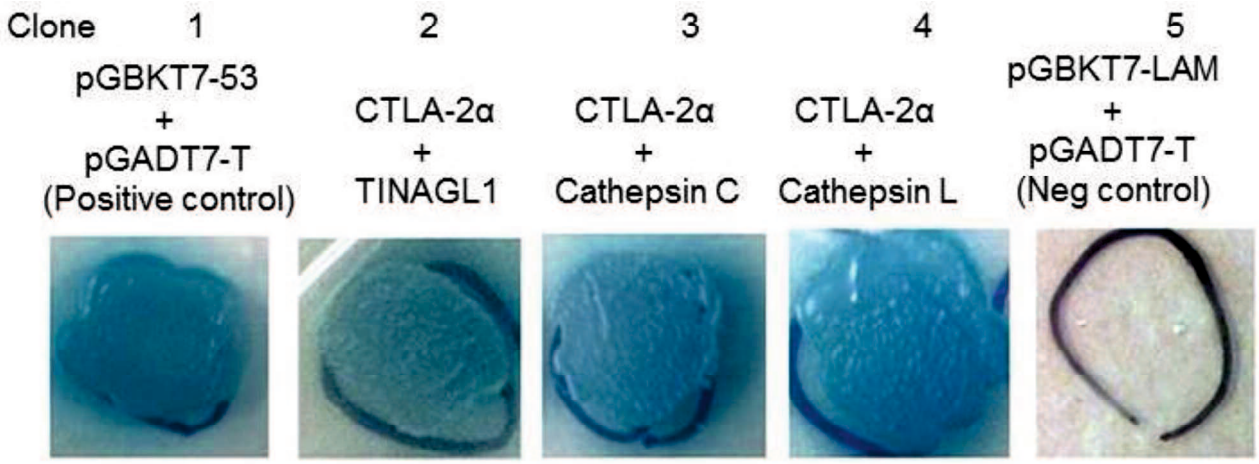


Figure 3A: Cathepsin L, cathepsin C, and TINAGL1 are proteins that interact with CTLA-2α. A. Three interactive proteins with CTLA-2α: cathepsin L, cathepsin C, and TINAGL1, were identified by yeast two-hybrid analysis. Clone 1 was a positive control (pGBKT7-53 and pGADT7-T). Clones 2, 3, and 4 were TINAGL1, cathepsin C, and cathepsin L co-transformed with CTLA-2α, respectively. Clone 5 was a negative control (pGBKT7-LAM and pGADT7-T).

Figure 3B. **The alignment of the interactive proteins,-cathepsin L, cathepsin C, and TINAGL1.** Highly conserved amino acid residues are shown in red. The position of the cysteine-replacing serine residue is marked by a box. GenBank accession numbers: cathepsin L: NP034114; cathepsin C: BC067063; TINAGL1 (Tubulointerstitial nephritis antigen-like 1 precursor) NP446034.

Figure 3C: Alignment between CTLA-2α and Pro region of cathepsin L. Highly conserved amino acid residues are shown in red

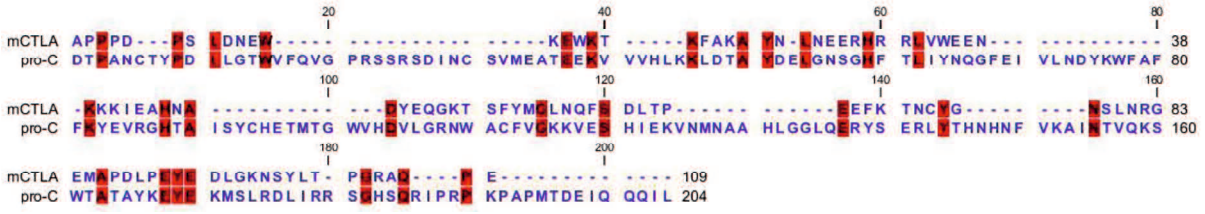


Figure 3D: Alignment between CTLA-2α and Pro region of cathepsin C. Highly conserved amino acid residues are shown in red

II. 3.3. CTLA-2α interacts with cathepsin L, cathepsin C and TINAGL1 in tissue

Detection of CTLA-2α/cathepsin L and CTLA-2α/cathepsin C complexes in the mouse uterus

A previous study demonstrated that CTLA-2α was highly expressed in the placenta (Campo et al., 1996). Therefore, pregnant mouse uteri were chosen to determine the interaction capability of CTLA-2α with cathepsin L and cathepsin C. CTLA-2α was co-immunoprecipitated with cathepsin L and cathepsin C from mouse uterus homogenates. Figure 5 shows SDS-PAGE of two combination experiments. Western blot analysis using anti-cathepsin L Ab clearly showed a single protein band of approximately 24 kDa (Fig. 5; lane 1). By comparing with the negative control (lane 2), the appearance of the single band indicated that CTLA-2α interacted with cathepsin L. When the same sample was probed with anti-cathepsin C Ab, only a single band of 25 kDa was detected (lane 3), which was not detected in the negative control (lane 4). This clearly indicated that the cathepsin C protein was also recovered from the Co-IP assay. Thus, these results indicated that cathepsin L and cathepsin C interacted with CTLA-2α in the mouse uterus.

Detection of the CTLA-2α/TINAGL1 complex

Isolation of CTLA-2α/TINAGL1 complex from pregnant mouse uteri by Co-IP was unsuccessful. Therefore, pull-down assay techniques was used to examine the interaction between CTLA-2α and TINAGL1on HEK293T cells that overexpressed TINAGL1 proteins. TINAGL1 is known to be actively secreted (Wex et al., 2001) and part of CTLA-2α is also secreted extracellularly. Therefore, recombinant CTLA-2α was added to the culture medium 24h after transfection before the pull-down assay. Figure 6 shows the result of two independent experiments, Western blot analysis

before and after the assay. As shown in Figure 6, a single protein band corresponding to TINAGL1 could be detected in lanes 1 and 3 after probing with anti-TINAGL1 Ab. That band was similar with the band appear in lane 3 which The absence of that band in lane 2, in which CTLA-2α was not added to the culture medium, indicated that the CTLA-2α/TINAGL1 complex was successfully pulled down from the culture medium.

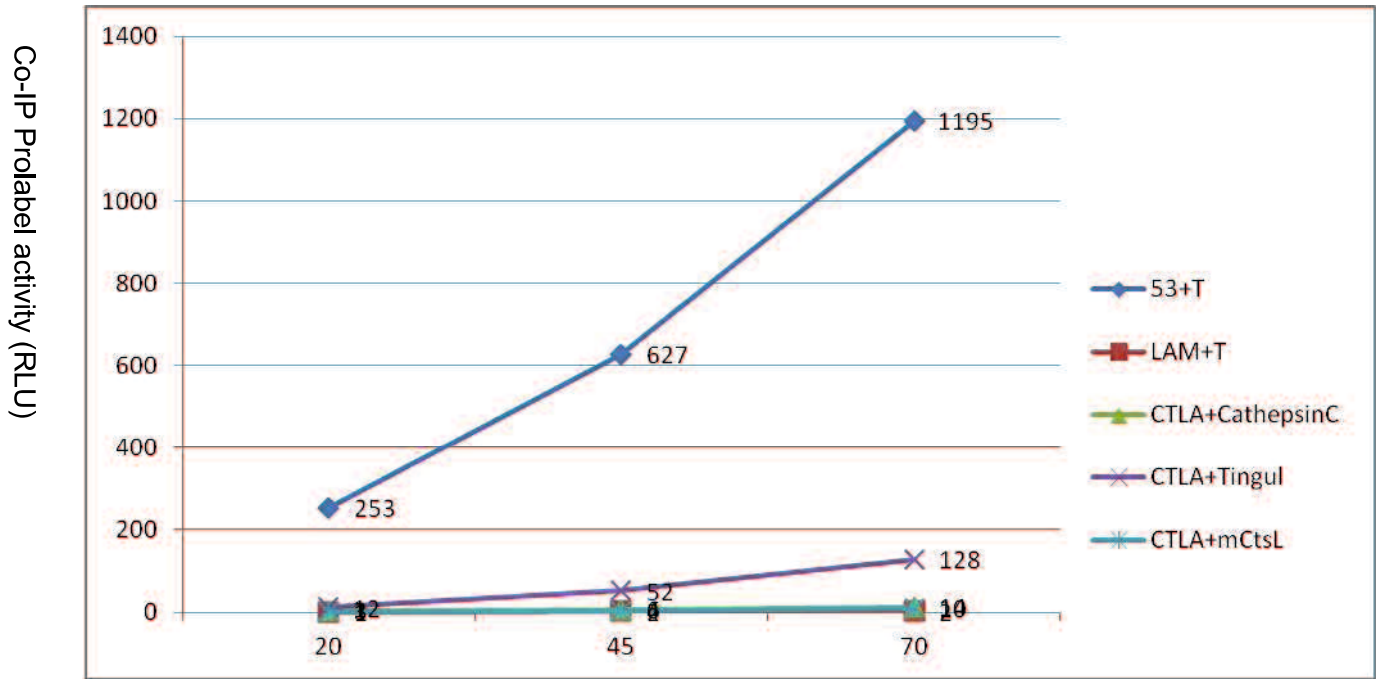


Figure 4: Detection of Prolabel activity from bait-prey complexes. HEK293 cells co-transfected with AcGFP- tagged CTLA-2α and Prolabel-tagged preys (cathepsin L, cathepsin C or TINAGL1) were immuneprecipitated with the anti-AcGFP1 Ab and assayed for Prolabel activity.

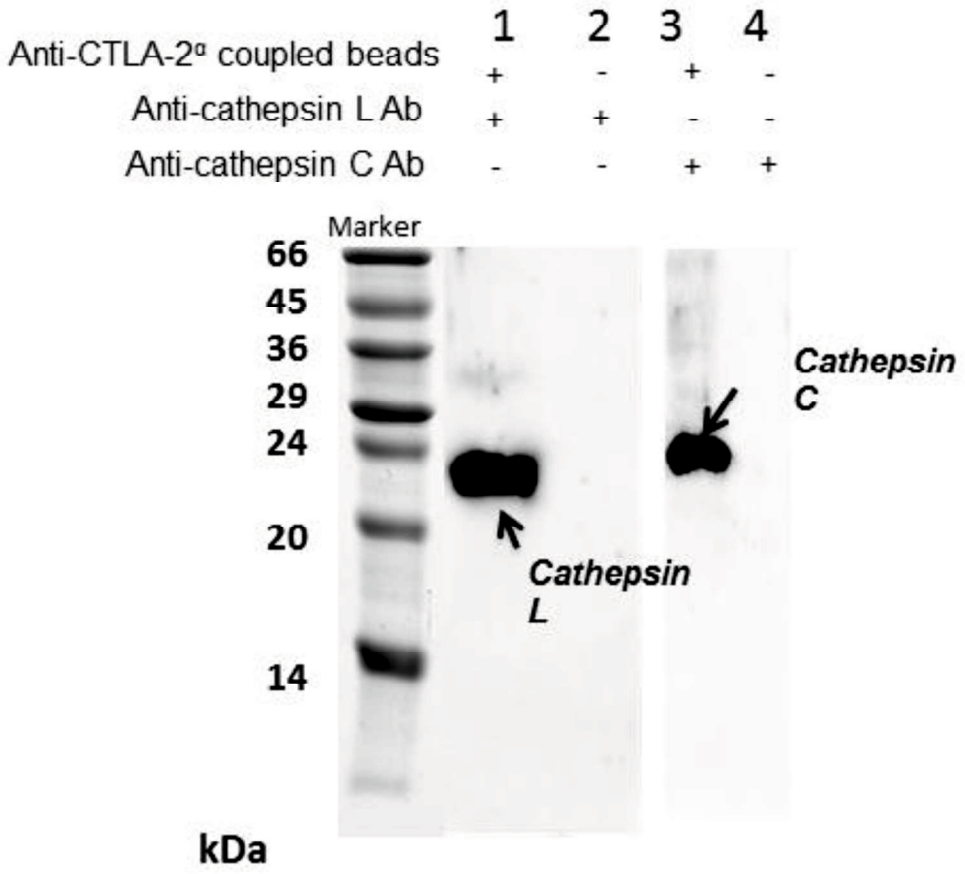


Figure 5: Isolation of CTLA-2α/cathepsin L and CTLA-2α/ cathepsin C complexes from the mouse uterus. A 10 dpc mouse uterus extract was subjected to Co-IP using Anti-CTLA-2α IgG coupled beads (lanes 1 and 3) or uncoupled beads (lanes 2 and 4). The recovered proteins were subjected to 12% SDS-PAGE. Proteins immunoreactive to the anti-cathepsin L (lanes 1 and 2) and anti-cathepsin C (lanes 3 and 4) antibodies were visualized.

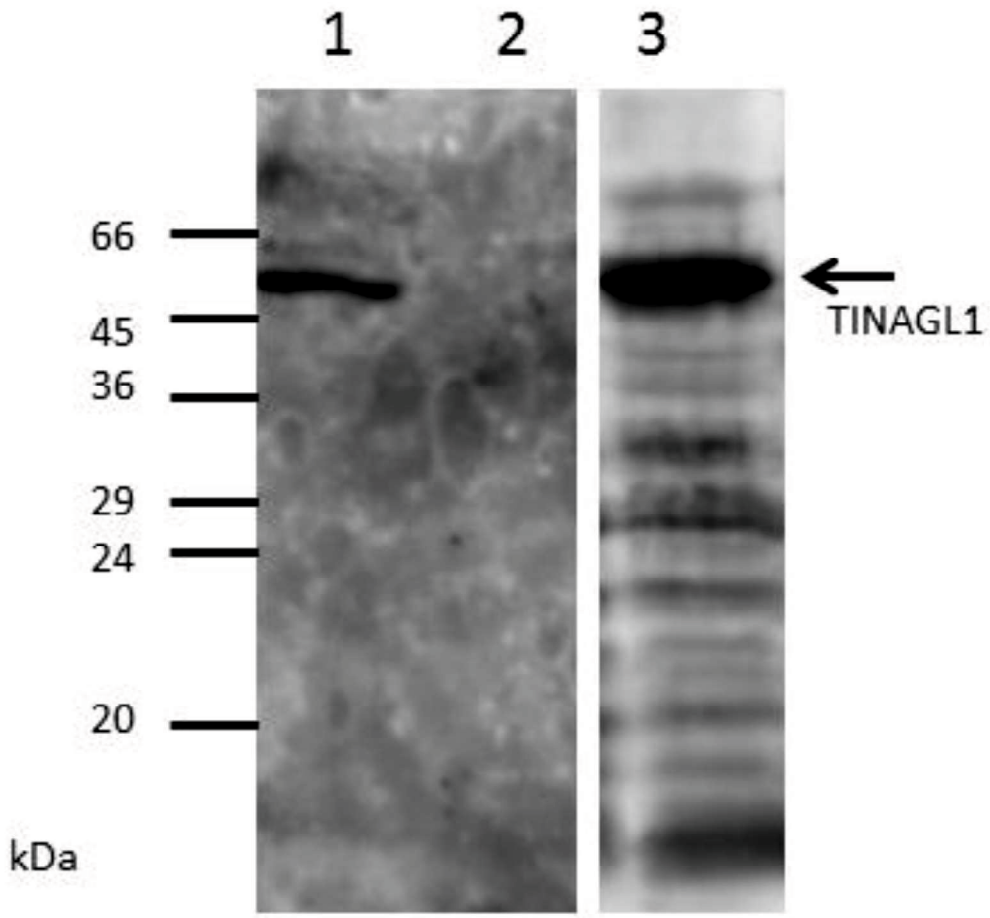


Figure 6: Pull-down of the recombinant CTLA-2α/TINAGL1 complex from the culture medium of HEK293T cells transfected with pFU.TINAGL1. The recombinant His-CTLA-2α protein (lane 1) or PBS (lane 2) was added to the culture medium 24h after transfection, followed by a 7-h culture. The medium was collected and subjected to a pull-down assay using the His-tag resin. The recovered proteins were subjected to 12% SDS-PAGE. Lane 3; culture medium before the pull-down assay. Proteins immunoreactive to the anti-TINAGL1 Ab were visualized.

Study II

Characterization of the interactions

between CTLA-2α and other proteins

1. LITERATURE REVIEW

Over the past decades, several cysteine proteinase inhibitor proteins homologous to the proregion of papain like cysteine proteinases have been reported (Yamamoto et al., 2002): bombyx cysteine proteinase inhibitors (BCPIs) (Yamamoto et al., 1999; Kurata et al., 2001), cytotoxic T-lymphocyte antigen-2s (CTLA-2s) (Delaria et al.; 1994; Kurata et al., 2003). BCPI (BCPI-a and b) are cysteine proteinase inhibitor proteins, which are found in the hemolymph of the silkmoth Bombyx mori and are homologous to the proregion of certain cysteine proteinases including *Bombyx* cysteine proteinase and are highly selective inhibitors of cathepsin L-like cysteine proteinases (Yamamoto et al., 1999; Kurata et al., 2001). CTLA-2s genes are mapped to the C1 band of mouse chromosome number 13, which encodes 109 amino acid residues with additional amino-terminal hydrophobic amino acid sequences, the transcripts of which were originally found in mouse activated T-lymphocytes and mast cells. The amino acid sequences of CTLA-2 are highly homologous to those of mouse cathepsin L. Recombinant CTLA-2s have been shown to be potent, competitive inhibitors of cathepsin L-like cysteine proteases. We previously identified the essential amino acid residues of CTLA-2α necessary for its inhibitory potency (Deshapriya et al., 2007). Three Trp residues (W12, W15, and W35) in the α1/α2-helixes, which form the hydrophobic core structure between the first and second α-helix, significantly contributed to inhibition. Another essential amino acid residue was shown to be cysteine 75 (C75), which is located in the immediate vicinity of the CTLA-2α region, and is interactive with the active-site cleft of the enzyme. We also suggested the possibility of disulfide bonding between CTLA-2α and the enzyme. In the chapter, we attempted to elucidate the functional

roles of the cysteine residue in more detail and clarify the interacting characterization between CTLA-2α and cathepsin L, cathepsin C.

2. MATERIAL AND METHODS

Expression of CTLA-2α in HEK293T cells.

Full-length cDNA encoding CTLA-2α was cloned into a pFUΔss plasmid (modified from pFUSE-hIgG2-Fc1). HEK293T cells (human embryonic kidney cells) were cultured and maintained in DMEM medium supplemented with 10% fetal calf serum. Transfection was performed using Lipofectamine LTX and PLUS according to the instructions provided by the manufacturer. After 48h culturing, the whole cell lysate and culture medium were collected and subjected to SDS-PAGE. Western blotting was carried out according to the method described previously (Luziga et al., 2008).

Monomer- Dimer conversion of CTLA-2α.

The dimeric form of CTLA-2α (150 μg) was incubated for 15 min at 37°C in 500 μl of the reducing buffer (150 mM NaCl, 1 mM EDTA, 50 mM DTT, and 100 mM Tris-Cl, pH 7.4). After incubation, the buffer was replaced by equilibration buffer (150 mM NaCl, 1 mM EDTA, 100 mM Tris-Cl, pH 8.2) containing or not containing 2 mM GSSG using a PD MiniTrap G-25 column. The samples were further incubated at 4°C overnight. The inhibition assay and SDS-PAGE analysis were then performed. For the inhibition assays towards CtsL, 30 μl of the CTLA-2α fraction was added to the assay buffer (500 μl) as described above.

Isolation of CTLA-2α/cathepsin L complex.

Cathepsin L and CTLA-2α were preincubated in 500 μl of preincubation buffer (1 mM EDTA, 8 mM cysteine, and 100 mM sodium acetate, pH 5.0) at 37°C. After 5

min. the buffer was quickly exchanged with buffer containing 150 mM NaCl, 1 mM EDTA, and 100 mM Tris-Cl, pH 7.4 by gel filtration using a PD MiniTrap G-25 column. The sample was then further incubated for 20 min at 37°C. In order to isolate the CTLA-2α/cathepsin L complex, an aliquot of the incubated sample was applied to a His-bind resin (Dynabeads TALON). The unbound proteins were removed by washing and the target proteins containing CTLA-2α/cathepsin L complex were specifically eluted according to the manufacturer's instructions. For measurements of cathespin L enzyme activity, an aliquot of the complex fraction was incubated in 0.1 M sodium acetate (pH 5.5) or 0.1 M sodium phosphate (pH 7.4) containing 1 mM EDTA, 8 mM cysteine. The enzyme reaction was started by the addition of Z-Phe-Arg-MCA (10 μM). The progress curves were monitored continuously for 5 min at 37°C at excitation and emission wavelengths of 370 and 460 nm with a spectrofluorometer (model F2000, Hitachi). SDS-PAGE and immunoblotting analyses were performed as described previously (Luziga et al., 2008). Affinity purified rabbit anti- CTLA-2α and anti-cathepsin L antibodies were prepared as described previously (Luziga et al., 2009).

Simple cell-based fluorescence assay for cathepsin C activity in live cells

Cathepsin C activity was detected as previously described (Thong et al., 2011) with some modifications. Cos-7 cells (derived from the kidney of the African Green monkey) were cultured in DMEM (Wako) supplemented with 10% fetal bovine serum at 37°C in a humidified atmosphere containing 5% CO_2. The cDNA encoding cathepsin C was cloned into the pBApo-CMV Neo DNA plasmid (Takara, Osaka, Japan) to generate pBApo.Cathepsin C, which was then transfected into Cos-7 cells. Cathepsin C-expressing Cos-7 cells were selected on neomycin (G-418 sulfate

solution, WAKO). After a 1-week selection, the transient cathepsin C-expressing cells were sequentially transfected with the pFU.CTLA-2α plasmid, which contained cDNA encoding CTLA-2α. The activity of cathepsin C in live cells was examined using a fluorogenic, cell-permeant, peptide substrate glycyl-phenylalanyl- amino-fluoro-coumarin (GF-AFC, Promega Corporation, WI, USA). The cells were washed in PBS, followed by a 1h incubation with PBS containing GF-AFC (100μM), DMSO (1%), and 2 min with DAPI (4',6-diamidino-2-phenylindole) for nuclear staining. Images were captured using a BZ-9000E HS all-in-one Fluorescence Microscope (KEYENCE, Japan) at Ex λ400nm and Em λ505nm. The original Cos-7 cells and cathepsin C-expressing Cos-7 cells were used as negative and positive controls, respectively.

3. RESULTS

3.1. Expression of CTLA-2α from HEK293T cells

The CTLA-2α protein was overexpressed in HEK293T cells, which demonstrated the existence of both intra- and extracellular functions (Fig. 7). The expressed protein could be present as the monomeric and dimeric form. Most of the CTLA-2α secreted extracellularly was identified as the dimeric form.

3.2. Monomer – Dimmer conversion of CTLA-2α

As described above, CTLA-2α can be present as either the monomeric or dimeric form. CTLA-2α has one cysteine residue (Cys75). In a previous study, we constructed mutant CTLA-2αs by replacing the cysteine residue with an alanine or serine residue (C75A, C75S). The dimeric form of CTLA-2α was no longer detected by SDS-PAGE of such mutant proteins, suggesting that the dimeric form of CTLA-2α resulted from the formation of an intermolecular disulfide bond between monomers. Interestingly, the inhibitory activity of the cysteine mutants (C75A, C75S) was markedly reduced. As a further investigation of the functional roles of the cysteine residue, we first isolated the dimeric form of CTLA-2α by separating it from the monomeric form (Figure 8, lane 2). It was fully inhibitory against CtsL. By treating it with the strong reducing reagent, DTT, the dimeric form lost all its inhibitory activity, and was completely converted to the monomeric form (Figure 8, lane 3). The preparation was then subjected to gel filtration to remove DTT, and further incubated in the absence or presence of L-glutathione (oxidized, GSSG). After overnight incubation, a part of the monomeric form was reassembled to the dimeric form, and recovered trace amounts of inhibitory activity (Figure 8, lane 4). Interestingly, in the presence of GSSG, CTLA-2α fully recovered its inhibitory activity although its

molecular form remained mostly as the monomeric form (Figure 8, lane 5). The possibility of inhibition of cathepsin L by GSSG could be omitted because GSSG (up to 1 mM) did not exhibit inhibitory activity towards cathepsin L in the present assay system (data not shown), and a final concentration of GSSG of the assay mixture containing CTLA-2α treated with GSSG was 0.12 mM.

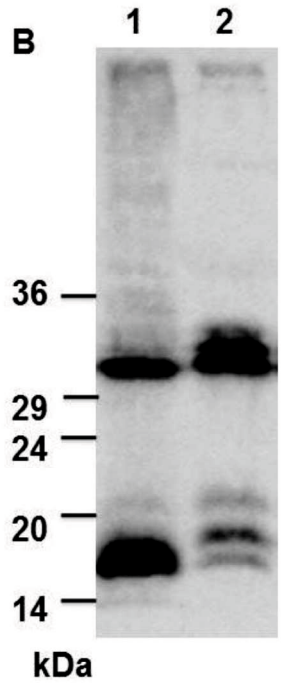


Figure 7. CTLA-2α expressed in HEK293T cells. Samples were subjected to SDS-PAGE using 12 % polyacrylamide gels in the absence of 2-mercaptoethanol, and analyzed by Western blotting using an anti- CTLA-2α antibody. Lane 1, cell extract; lane 2, medium.

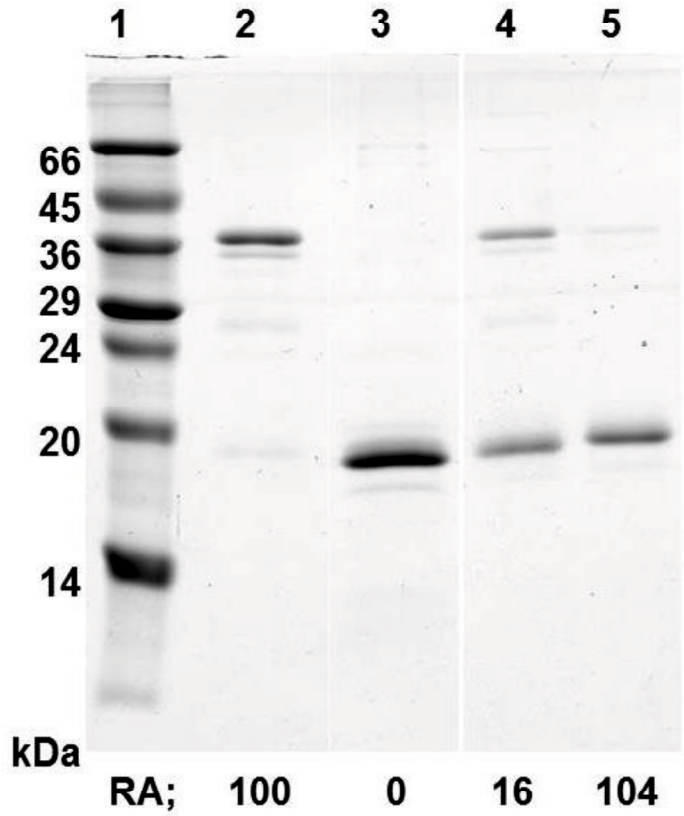


Figure 8. Dimer-monomer conversion of CTLA-2α. SDS-PAGE using 15% polyacrylamide gels was performed in the absence of 2-mercaptoethanol. Proteins were stained with Coomassie brilliant blue. Lane 1, protein molecular weight standards; lane 2, dimeric form of CTLA-2α; lane 3, sample of lane 2 treated with 50 mM DTT; lane 4, sample of lane 3 further incubated in the absence of DTT; lane 5, sample of lane 3 further incubated in the absence of DTT and in the presence of 2 mM GSSG. RA, relative inhibition activity towards cathepsin L, activity of the dimeric form of CTLA-2α as 100%.

3.3. Identification of the CTLA-2α/ cathepsin L complexes conjugated with a disulfide bond in vitro

Since cathepsin L is a cysteine protease having an essential cysteine residue at its active site, we next attempted to isolate the CTLA-2α/cathepsin L complex conjugated with a disulfide bond. CTLA-2α and/or cathepsin L were firstly preincubated for a short time in an acidic buffer (pH 5.0) containing cysteine to activate cathepsin L and allow the interaction between CTLA-2α and cathepsin L. The buffer was quickly exchanged with a neutral buffer (pH 7.4) not containing cysteine. After incubation, CTLA-2α and CTLA-2α conjugated proteins were recovered by precipitation with the His-bind resin from the incubation mixture. Figure 9 shows SDS-PAGE of several combination experiments. The His-bind resin specifically recovered CTLA-2α, but not cathepsin L (Figure 9, lanes 3, 4, 8, 9). When CTLA-2α was incubated with cathepsin L, several additional protein bands could be seen, indicating that these proteins were conjugated with CTLA-2α (lanes 2, 7). Western blot analysis using an anti-cathepsin L antibody clearly showed that among these proteins, the protein with a molecular weight of 24,000 was cathepsin L (lanes 2, 12). The lack of a band corresponding to cathepsin L in the incubation with the CTLA-2α mutant, C75S suggested that CTLA-2α was conjugated with cathepsin L by disulfide bonding (lanes 5, 10). Furthermore, the lack of a similar band was also observed in the incubation of CTLA-2α with cathepsin L lacking an active site cysteine residue (lanes 6, 11). The CTLA-2α/cathepsin L complex behaved as a heterogeneous molecular form in SDS-PAGE without 2-mercaptoethanol (lanes 7, 13). To ascertain whether the CTLA-2α/cathepsin L complex retains its enzyme activity or not, the cathepsin L activity of the isolated complex was continuously measured (Fig. 10). The complex did not exhibit enzyme activity even in the acidic

condition (pH 5.5). After dissociation of the complex by treatment with DTT, significant and pH dependent cathepsin L activities were measured.

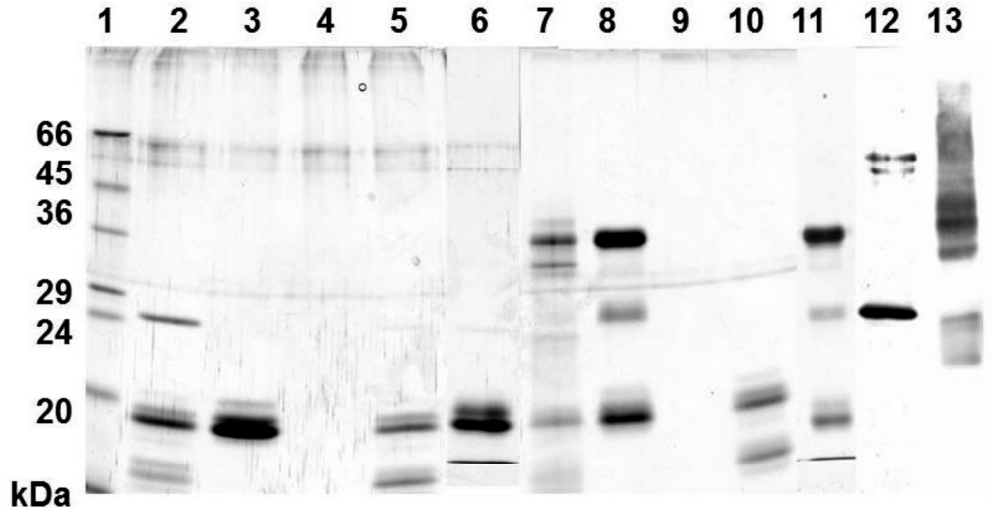


Figure 9. Isolation of the CTLA-2α/cathepsin L complex. SDS-PAGE using 12% polyacrylamide gels was performed with (lanes 1 – 6, 12) or without (lanes 7 – 11, 13) 2-mercaptoethanol. Proteins were stained with silver nitrate reagent (lanes 1 – 11). Proteins immunoreactive to the anti-cathepsin L antibody were visualized (lanes 12, 13). Lane 1, protein molecular weight standards; lanes 2, 7, 12, 13, CTLA-2α/ cathepsin L; lanes 3, 8, CTLA-2α; lanes 4, 9, cathepsin L: lanes 5, 10, CTLA-2α (C75S)/ cathepsin L; lanes 6, 11, CTLA-2α/ cathepsin L (E-64 treated).

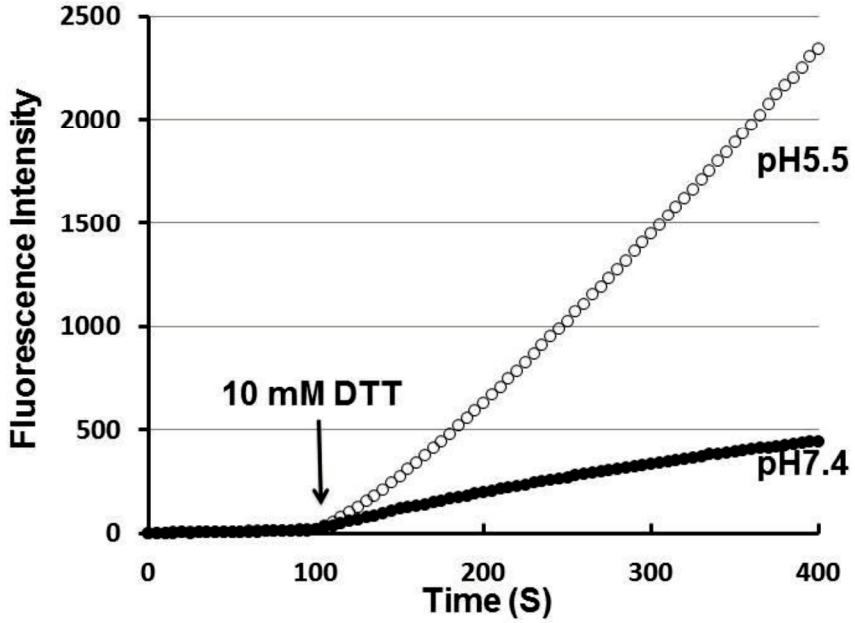


Figure 10: Progress curves for the cathepsin L activity of the CTLA-2α/cathepsin L complex. The progress curves were continuously monitored. The arrow indicates the time when 10 mM DTT was added to the reaction mixture.

3.4. Identification of the CTLA-2α/ cathepsin L, CTLA-2α/ cathepsin C complexes conjugated with a disulfide bond from the tissue

Cathepsin L is a cysteine protease that has an essential cysteine residue at the active site. CTLA-2α also contains a cysteine residue (C75), which acts as a disulfide form and is engaged in its inhibitory potency (Deshapriya et al., 2010). As described above, the recombinant CTLA-2α/cathepsin L complex has been isolated *in vitro* and CTLA-2α may inhibit cathepsin L-like cysteine protease by oxidizing the active thiol residue of the enzyme with its own thiol residue. Therefore, we attempted to detect the CTLA-2α/cathepsin L complex conjugated with a disulfide bond *in vivo*. The proteins recovered from Co-IP were subjected to SDS-PAGE with or without 2-mercaptoethanol in the loading buffer and analyzed by western blotting using anti-cathepsin L Ab and anti-CTLA-2α Ab. When probed with anti-cathepsin L Ab (Fig. 11, lanes 1 - 3), a major band corresponding to cathepsin L was detected in the presence of 2-mercaptoethanol (lane 1). Three additional bands with higher molecular weights were detected in the absence of 2-mercaptoethanol (lane 3). Stripping and re-probing of the membrane with anti-CTLA-2α Ab (lanes 4 – 6) led to the appearance of a protein band that corresponded to the molecular weight of the complex (lane 6), which suggested that one of the additional bands was the CTLA-2α/ cathepsin L complex conjugated with a disulfide bond. The same method was applied to the sample for cathepsin C. A similar band pattern to that of cathepsin L was also detected, which suggested that cathepsin C was also conjugated with CTLA-2α by disulfide bonding (Fig.12).

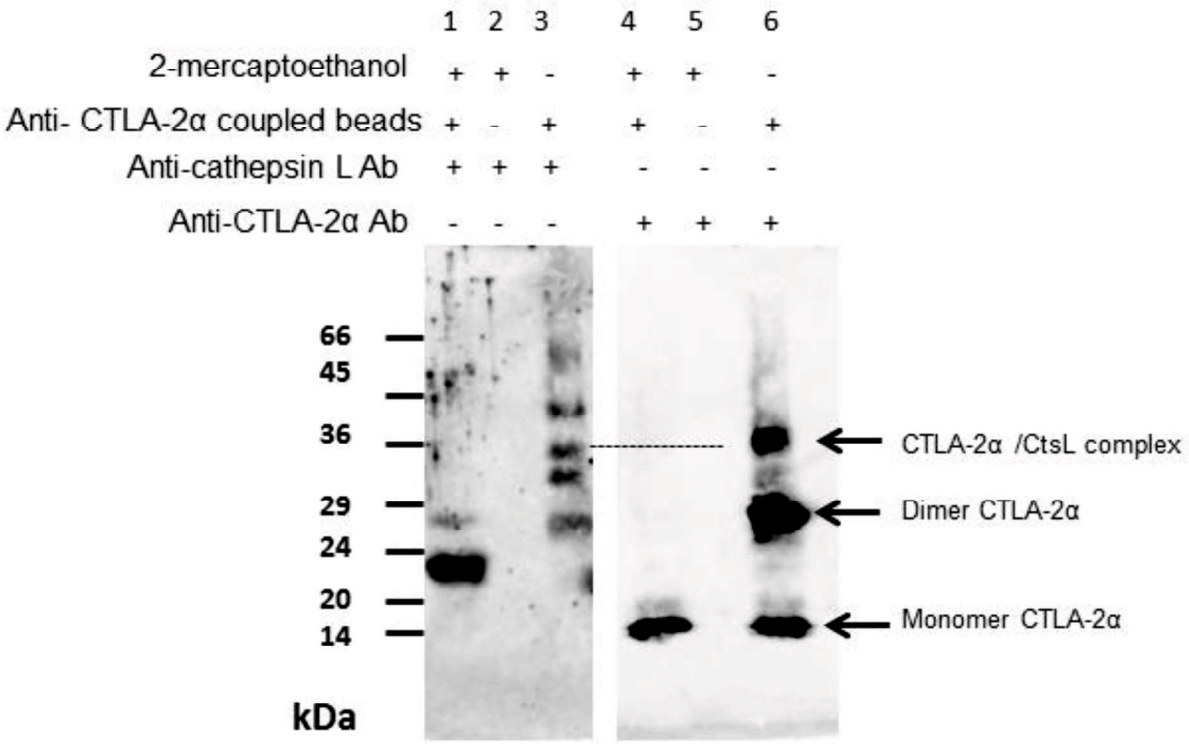


Figure 11. Isolation of the CTLA-2α/cathepsin L complex conjugated with disulfide bonds from tissue. A 10 dpc mouse uterus extract was subjected to Co-IP using Anti-CTLA-2α coupled beads, as shown in figure 5. The recovered proteins were subjected to 12% SDS-PAGE with (lanes 1, 2, 4, and 5) or without (lanes 3 and 6) 2-mercaptoethanol. Proteins immunoreactive to the anti-cathepsin L (lanes 1, 2, and 3) and anti-CTLA-2α (lanes 4, 5, and 6).

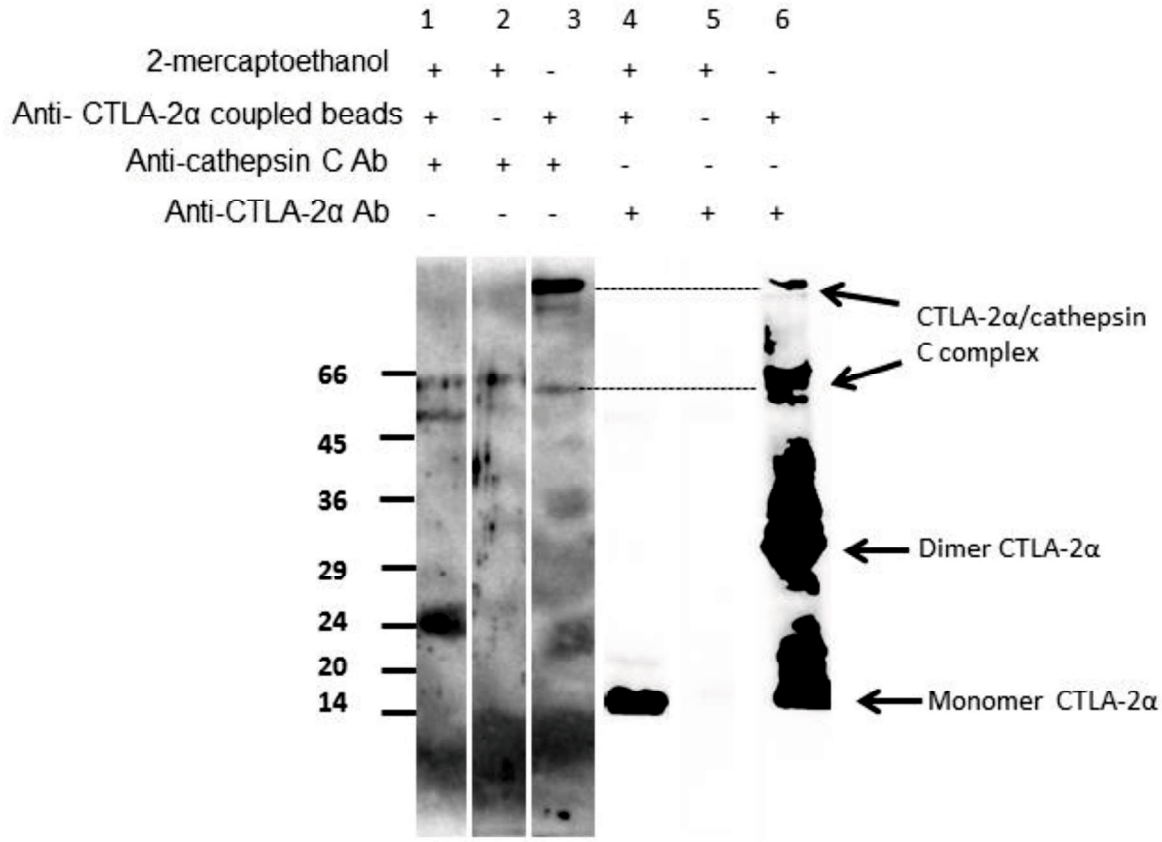


Figure 12. Isolation of the CTLA-2α/cathepsin C complex conjugated with disulfide bonds from tissue. A 10 dpc mouse uterus extract was subjected to Co-IP using Anti-CTLA-2α coupled beads, as shown in figure 5. The recovered proteins were subjected to 12% SDS-PAGE with (lanes 1, 2, 4, and 5) or without (lanes 3 and 6) 2-mercaptoethanol. Proteins immunoreactive to the anti-cathepsin C (lanes 1, 2, and 3) and anti-CTLA-2α (lanes 4, 5, and 6).

3.5. CTLA-2α exhibited inhibitory activity to cathepsin C in cells

We attempted to detect the inhibitory activity of recombinant CTLA-2α toward purified mouse cathepsin C (R&D systems) using Gly-Arg-AMC as the substrate, and found no inhibitory activity up to 1 μM of CTLA-2α (data not shown). Thus, we switched to the simple cell-based fluorescence assay, which was designed to determine the activity of cathepsin C in live cells. Cos-7 cells were selected because they had low endogenous cathepsin C (Santilman, 2002). (Fig. 13A, B). As shown in Figure 13, green staining was strongly captured in cathepsin C-expressing Cos-7 cells (Fig. 13C). The green signal was observed in about 74% of the cells expressing cathepsin C. This fluorescence labeling was significantly reduced in Cos-7 cells transfected with both cathepsin C and CTLA-2α (Fig. 13E). These results demonstrated that CTLA-2α inhibited cathepsin C activity in live cells.

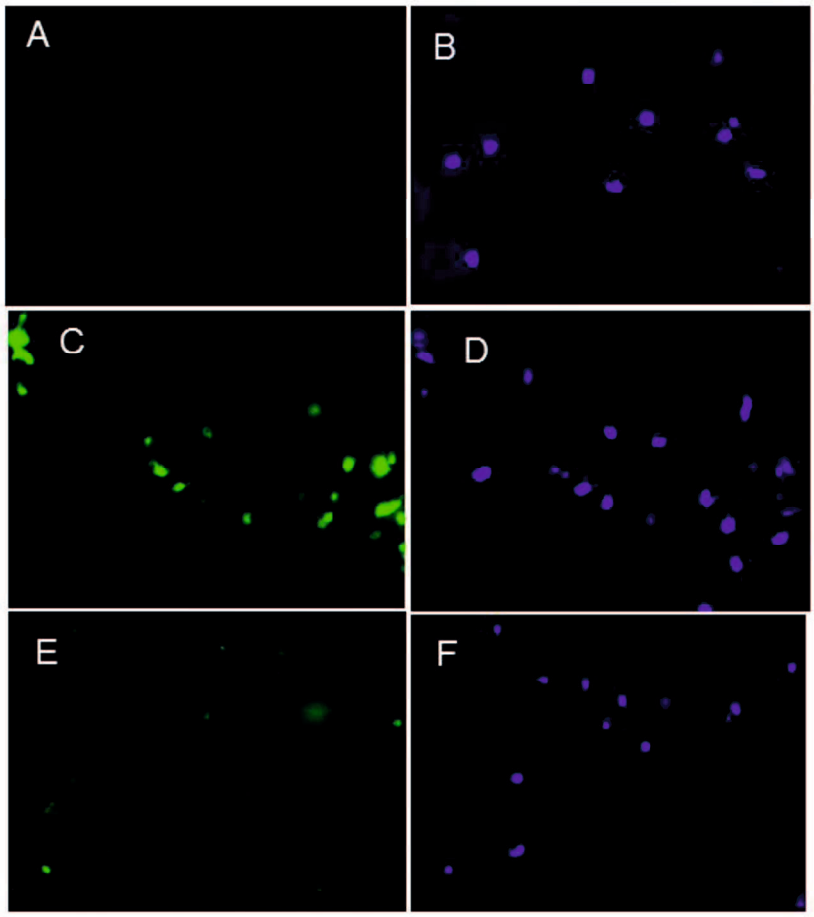


Figure 13. A simple cell-based fluorescence assay for detecting cathepsin C activity. Fluorescence imaging of cells incubated with GF-AFC substrate (A, C, E). Nuclear stained with DAPI (B, D, F). Cos-7 cells (A, B). Cos-7 cells were transfected with pBApo. Cathepsin C (C, D). Cos-7 cells were transfected with pBApo.Cathepsin C and pFU.CTLA-2α (E, F)

Study III

Co-localization of CTLA-2α with cathepsin L, cathepsin C and TINAGL1 in HEK293T cells and in the mouse placenta

1. LITERATURE REVIEW

CTLA-2α has been report to present in various organs, such as placenta (Campo et al., 1996), brain (Luziga et al., 2007), eye (Sugita et al., 2001) but little is known regarding the cellular expression and physiological function of CTLA-2α tissues and organs. Luziga et al (2007) reported the expression pattern of CTLA-2α mRNA in the mouse brain by in situ hybridization, demonstrating that the mRNA is preferentially enriched within various neuronal populations. That expression pattern suggests that CTLA-2α may have some roles related to memory and brain diseases.

At the cell level, CTLA-2α protein is mainly localized in dendritic and axonal compartments and to a lesser extent in neuronal cell bodies (Luziga et al., 2007). Similarly, strong staining was detected in basal dendrites of pyramidal neurons radiating through the stratum radiatum but was faint in pyramidal cell bodies. It can be hypothesised that CTLA-2α protein is synthesized in neuron cell bodies but is secreted to dendrites where it might be playing a role in regulating neuronal function. CTLA-2 α mRNA was mainly observed by Northern blot analysis in placenta and uterus and to a lesser extent in the lungs (Campo et al., 1996). CTLA-2α mRNA has also been demonstrated in mouse uterus during early pregnancy (Cheon et al., 2004). Interestingly, three proteins that interact with CTLA-2α present in placenta as well. Cathepsin L is shown to be present in large amounts in the placenta, six times more than adult kidney and liver (Hamilton et al., 1991). Two forms of cathepsin L, pro and matureL, both exist in the mouse placenta. Giant cells and spongiotrophoblast were identified to synthesize and secrete procathepsin L. The large amount of cathepsin L in placenta may involve with defined function of tissue invasion in the embryonic developmental process.

Cathepsin C is a cysteine protease involved in the processing of lysosomal cathepsins and the degradation of the intracellular protein (Methot et al., 2007). Cathespsin C performs important functions in many physiological and pathological progresses. However, its function is best characterized in the immune system. Cathepsin C transcript and protein both have been detected in placenta (Rao et al., 1997; Menkhorst et al., 2012). Both pro and mature forms of cathepsin C were observed in the decidua region of placenta. It is believed to play an important role in the implantation and placentation process.

The third protein - TINGAL1- interestingly also presents in the placenta. TINAGL1 is known as a matricellular protein that homologous to cathepsin B. However, physiological function and localization of TINAGL1 are still not clearly understood. In gestation, TINAGL1 has been expressed in extra-embryonic tissue and plays a physical and physiological role in embryo development at postimplantaion (Igarashi et al., 2009; Li et al., 2007).

Taken together all these data, we hypothesized that CTLA-2α may associate with its interactive proteins to perform some physiological functions in placenta. In this chapter, information on the distribution of CTLA-2α in the placenta is presented. The co-localizations of CTLA-2α and three interactive proteins have been examined.

2. MATERIAL AND METHODS

Expression constructs, cell culture and transfection

Full-length of CTLA-2α, Cathepsin L, Cathepsin C and TINAGL1 were cloned into pFUΔss plasmid (modified from pFUSE-hIgG2-Fc1) to generate pFU-F.CTLA2 and pFU-F.CtsL, pFU-F.CtsC, pFU-F.TINAGL.

HEK293T (Human Embryonic Kidney) cells were cultured in DMEM (Wako) supplemented with 10% fetal bovine serum at 37^0C in a humidified atmosphere containing 5% CO2. DNA transfection of HEK293 cells was carried out at 60% – 80% confluent using ScreenFect A (Wako)

Immunofluorescence staining and imaging of HEK293T cells line

To detect the co-localization of CTLA-2α and cathepsin L/cathepsin C/ TINAGL1 from the transfected cells, HEK293T cells plated on round coverslip and co-transfected with pFU-F.CTLA-2 and pFU-F.CtsL; pFU-F.CTLA-2 and pFU-F.CtsC ; pFU-F.CTLA-2 and pFU-F.TINAGL-1. After 24h, cells were washed twice with PBS and fixed in 4% paraformaldehyde. Cells were permeabilized with 0.2% Triton X-100 and further incubated with blocking buffer (PBS containing 1% Bovine Serum Albumin, 0.1% Triton x-100) for 30 minutes at RT to block non – specific binding. The cells were incubated with mixture of two diluted primary antibodies from difference host species (diluted in blocking buffer) for 24 hours in humid chamber at 4ºC. For negative control, cells were incubated with normal serums of the same species and same concentration as the primary antibodies. After incubation, the cells were washed (3 x 5 min) by PBS/ 0.1% Triton X-100, followed by incubation for 1 hour at RT with a mixture of Alexa Fluor 594 conjugated Goat anti-chicken IgY and

Alexa Fluor 488 conjugated Donkey anti-Rabbit IgG. At the end of incubation, the cells were washed (3 x 5 mins) in PBS/0.2% Triton X-100 and further incubated for 2 mins with DAPI. Finally, coverslip were washed by PBS and mounted.

Immunofluorescence labeling and imaging of tissue

Immunofluorescence analysis was performed to determine the distribution profile of CTLA-2α in the mouse placenta. Pregnant mice were purchased from Kyudo (Saga, Japan) and used for the experiment at 8, 9, 10, 14 and 17 days post coitum (dpc). Placenta tissues were snap-frozen, then sectioned at 15-μm and mounted onto silane-coated slides and stored at -80ºC until use. After thawing at RT, sections were rehydrated in PBS for 10 min and fixed in 4% paraformaldehyde. The sections were then treated with PBS containing 1% BSA and 5% of donkey serum to block non-specific binding. Sections were incubated in rabbit anti-CTLA-2α IgG for 24h in a humid chamber at 4ºC for the primary Ab reaction. Sections incubated in normal rabbit serum with the same concentration as the primary Abs were used as negative controls. After washing with PBS (5 min x 3 times), followed by incubation for 1h at RT with Alexa Fluor 488 conjugated donkey anti-rabbit IgG, washing in PBS/ 0.1 Triton X-100 (3 x 5 min) and a further incubation for 2 min with DAPI, sections were again washed with PBS and mounting. The expression of cathepsin L was examined in 10 dpc and 12 dpc mouse uteri with chicken anti-cathepsin L IgY and Alexa Fluor 594 conjugated goat anti-chicken IgY.

To confirm the co-localization of CTLA-2α and cathepsin L/cathepsin C/TINAGL1 *in vivo,* double immunostaining was performed on mouse uterus sections. The initial steps used to process tissues were the same as for single

labeling. However, the sections were incubated with a mixture of diluted primary Abs: rabbit anti-CTLA-2α IgG/chicken anti-cathepsin L IgY; chicken anti-CTLA-2α IgY/rabbit anti-cathepsin C IgG; chicken anti-CTLA-2α IgY/rabbit anti-TINAGL1 IgG. In the second Ab reaction, sections were treated with a mixture of Alexa Fluor 594 conjugated goat anti-chicken IgY and Alexa Fluor 488 conjugated donkey anti-rabbit IgG. Immunolabeling was analyzed using the BZ-9000E HS all-in-one Fluorescence Microscope (KEYENCE, Japan).

3. RESULTS

3.1. Expression of Cathepsin L, TINAGL1 and CTLA-2α in the co-transfected HEK293T cells

Further confirmation of the interaction between Cathepsin L, TINAGL1 and CTLA-2α were obtained by double immunofluorescence labelling of HEK293T cells co-transfected with pFU-F.CTLA-2 + pFU-F.CtsL and pFU-F.CTLA-2 + pFU-F.TINAGL vectors and stained cells were visualized by fluorescence microscopy (Fig.14). The results showed that CTLA-2α (Fig.14A; green) and Cathepsin L (Fig.14A; red) were uniformly distributed throughout the cells. Similarly, CTLA-2α (Fig.14B; red) and TINAGL1 (Fig.14B; green) can also be detected throughout the transfected cells. When the images were overlaid (Fig.14A & 14B; yellow), both Cathepsin L and TINAGL1 overlapped with CTLA-2α, suggesting that Cathepsin L and TINAGL1 interact with CTLA-2α in those cells.

3.2. Distribution of CTLA-2α and cathepsin L in the mouse placenta at various developmental stages

The distribution pattern of CTLA-2α in the mouse placenta was analyzed in transversal sections of the whole pregnant mouse uterus and placenta in the five difference stages of pregnancy. Before carrying out the immunofluorescence staining, Hematoxylin and Eosin stained sections has been used to identify each region (Fig. 15A). By immunofluorescence labeling, CTLA-2α displayed a region–specific localization, it was strongly detected in some placental regions, but was absent in others (Fig. 15B). At 8 and 9 dpc, the well-developed decidua (WD) and myometrium (MM) were highly stained by the anti-CTLA-2α Ab, whereas the region of

endometrium (ED) was weakly stained. CTLA-2α was consistently detected in the decidua basalis (DB) and metrial gland (MG), but not in the placental labyrinth (PL) at 10 dpc. By 14 and 17 dpc, CTLA-2α was highly detected in the DB, MG, and (MM). Almost blank labeling was observed when the anti-CTLA-2α antibody was replaced with normal rabbit serum. The specific spatial expression pattern and strong induction of expression suggested an essential role for CTLA-2α in the formation and development of a functional mouse placenta. The presence of cathepsin L in the placenta was then evaluated. The cathepsin L protein was highly expressed in the whole placenta including the labyrinth at 10 and 12 dpc (Fig. 15C). The signal was also observed in the PL and embryo, indicating that cathepsin L was expressed ubiquitously.

3.3. Co-localization of cathepsin L, cathepsin C, and TINAGL1 with CTLA-2α in the mouse placenta.

The co-localization of cathepsin L, cathepsin C, and TINAGL1 with CTLA-2α was evaluated in the placenta using double-immunofluorescence labeling. Figure 16 showed that cathepsin L was expressed ubiquitously in the uterus, whereas CTLA-2α, cathepsin C, and TINAGL1 were only detected in certain regions of the placenta. As shown in Figure 16A, cathepsin L (red) was clearly observed in the whole 10 dpc uterus including the embryonic region, while the CTLA-2α signal (green) was absent in the embryo and PL. This result suggested that CTLA-2α may be co-localized with cathepsin L in the DB and MG of the placenta. Co-localization was absent in other regions. As shown in Figure 16B, staining for cathepsin C (green) in the placenta at 10 dpc was weakly detected in the labyrinth, DB and MG. The co-localization of CTLA-2α and cathepsin C was observed in the DB and MG (yellow). TINAGL1 was

previously reported to be expressed in the post-implantation uterus. Therefore, double immunofluorescence analysis was carried out in 8 dpc uterus to identify the co-localization of CTLA-2α and TINAGL1. Figure 16C showed that the expression patterns of CTLA-2α (red) and TINAGL1 (green) were similar in the mouse uterus (8 dpc). They were strongly detected in the WD and MM regions (yellow). Weak co-expression was observed in the ED and no labeling was detected in the immature placental labyrinth.

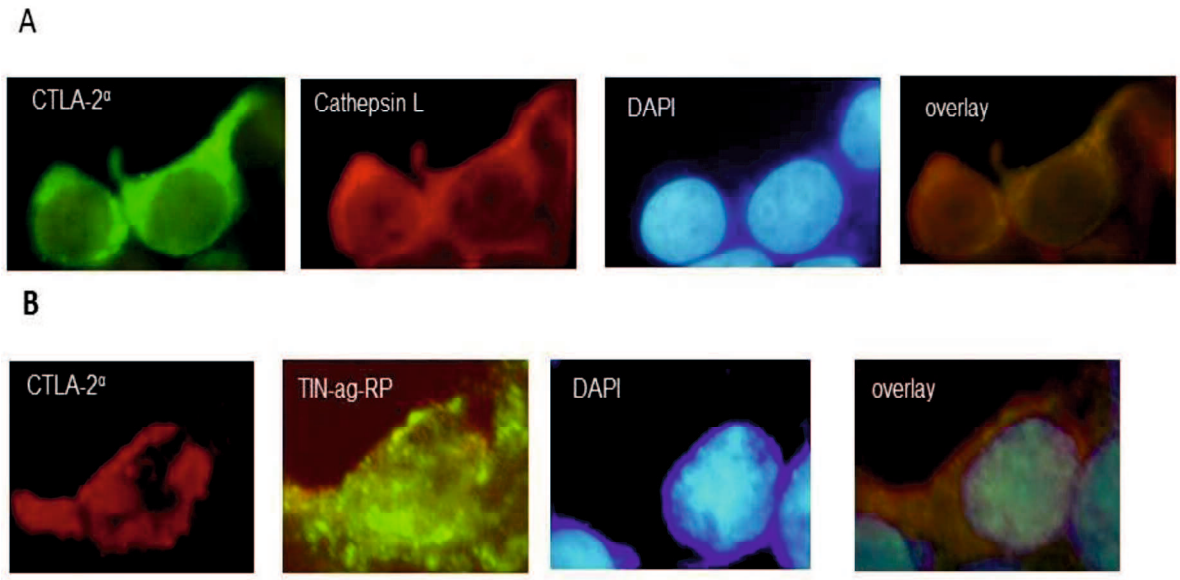


Figure 14. Co-localization of Cathepsin L, Cathepsin C, TINAGL1 with CTLA-2α in HEK293T cells. (**A**) CTLA-2α (green) and Cathepsin L (red) proteins were visualized by double-immunostaining. The overlay image shows region of signal overlap. (**B**) HEK293T cells were co-transfected with expression vectors for CTLA-2α and TINAGL1. Protein expression was detected using double-immunostaining. The two figures [CTLA-2α (red) and TINAGL1 (green)] have been merged (yellow).

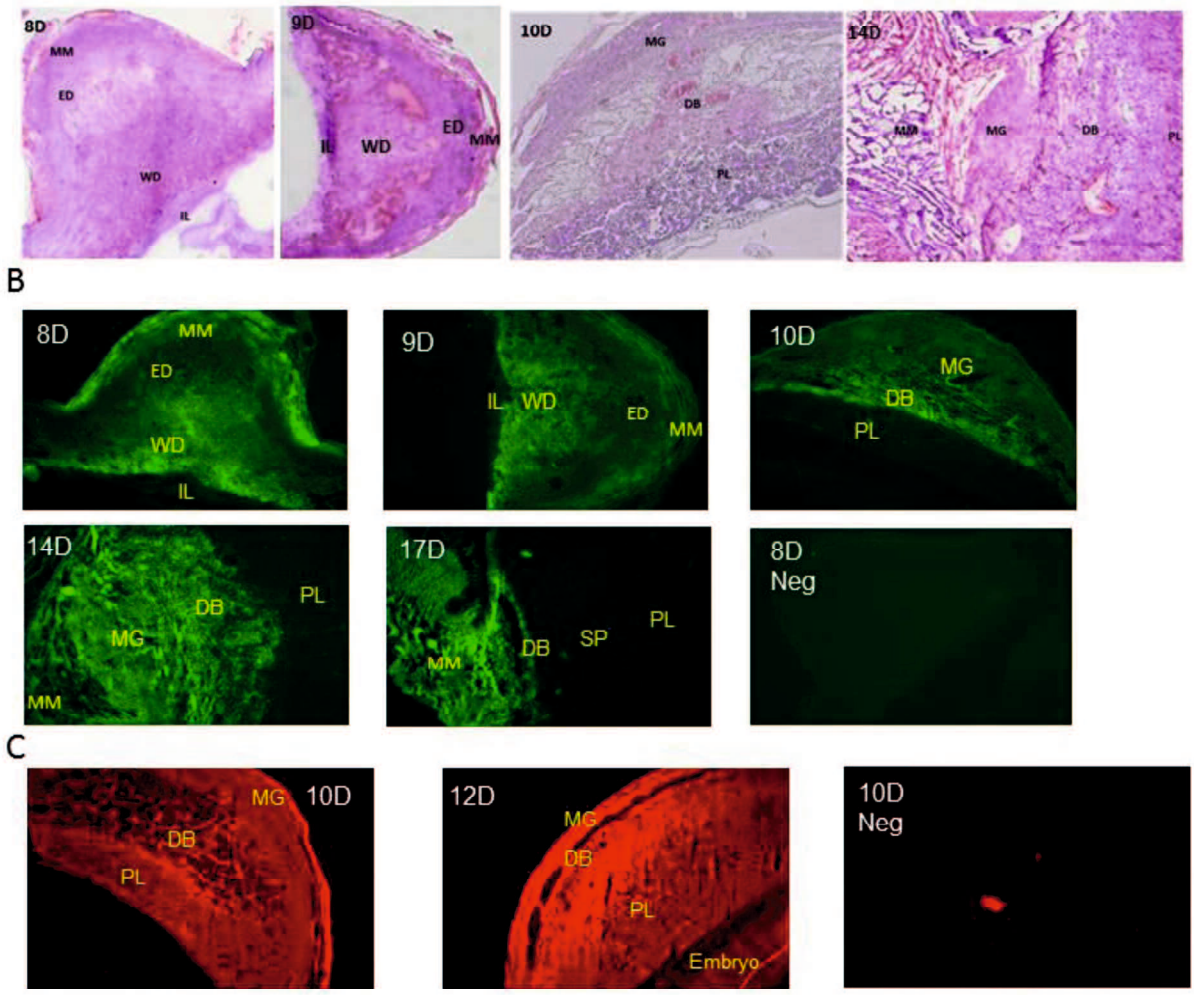


Figure 15. Distribution of CTLA-2α and cathepsin L in the mouse placenta at various placentation periods. **A**) Hematoxylin and Eosin staining was performed for the mouse uterus (8-10 dpc) and mouse placenta (14 dpc). Immunofluorescence labeling was performed for the mouse uterus (8-10 dpc) and mouse placenta (14-17 dpc). **B**) Immunoreactivity for CTLA-2α (green) in the mouse placenta at 8 dpc (8D), 9 dpc (9D), 10 dpc (10D), 14 dpc (14D), 17 dpc (17D). Neg; negative control stained with normal rabbit serum (8D). **C**) Immunoreactivity for cathepsin L (red) at 10 dpc (10D), 12 dpc (12D). Neg; negative control stained with normal chicken IGY (10D). Immature labyrinth (IL), placental labyrinth (PL), spongiotrophoblast (SP), decidua basalis (DB), well-development decidua (WD), endometrium (ED), metrial gland (MG), and myometrium (MM).

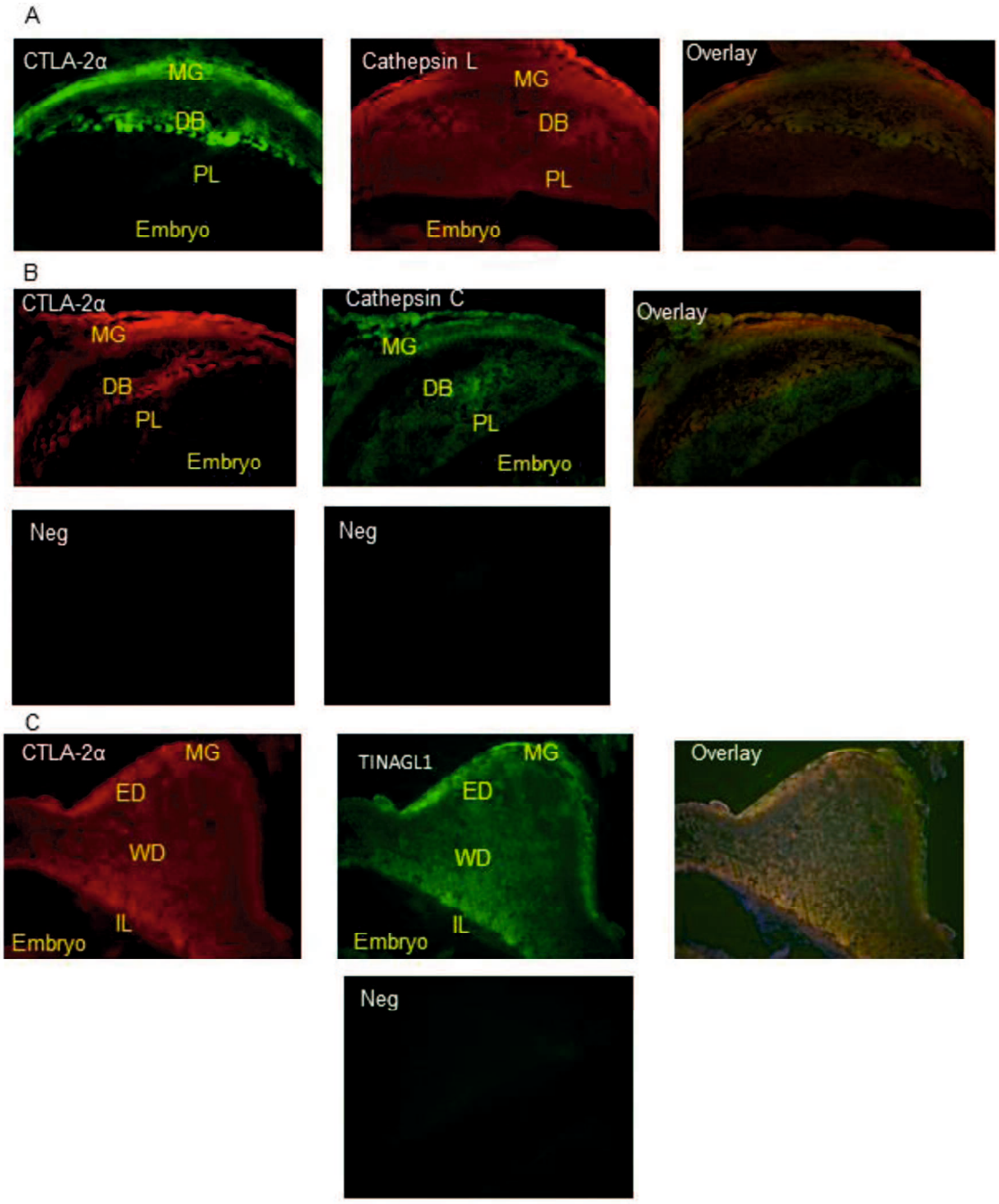


Figure 16. Co-localization of cathepsin L (A), cathepsin C (B), and TINAGL1 (C) with CTLA-2α in 8 and 10 dpc mouse placenta. (**A**) The immunoreactivities of CTLA-2α (green) and cathepsin L (red) were observed. Two Abs (rabbit anti-CTLA-2α IgG and chicken anti-Cathepsin L IgY) were mixed and used as primary Ab. (**B**) CTLA-2α (red) and cathepsin C (green) immunoreactivities were detected. Two Abs (chicken anti- CTLA-2α IgY and rabbit anti-Cathepsin C IgG) were mixed and used

as primary Ab. A mixture of normal chicken IGY and normal rabbit serum was used as the negative control (Neg 1&2). **C**) CTLA-2α (red) and TINAGL1 (green,) immunoreactivities were observed. Two Abs (chicken anti- CTLA-2α IgY and rabbit anti-TINAGL1 IgG) were mixed and used as primary Ab. Normal rabbit serum was used as a negative control (Neg).

Discussion

Interaction between CTLA-2α and cathepsin L

Yeast two-hybrid analysis led to the discovery of the interaction between CTLA-2α and cathepsin L. This result was consistent with our previous findings in which recombinant CTLA-2α selectively inhibited cathepsin L- like cysteine proteinase (Kurata, 2003). In the present study, the CTLA-2α/cathepsin L complex was isolated from pregnant uteri, thereby confirming that CTLA-2α interacted with cathepsin L under physiological conditions. Results of this study were compared with the finding of the previous work in which CTLA-2β/cathepsin L complex was isolated from day 7 pregnant uteri extracts but not in 10 pregnant uteri (Campo, 1996). However, CTLA 2α/cathepsin L complex was detected in day in 10 pregnant uteri in this study.

CTLA-2α contains one cysteine residue, C75, in the sequence, rendering the formation of a dimer by an intermolecular disulfide bond between the monomers. In the present study, CTLA-2α was shown to be present as such a dimer in vitro and also possibly under physiological conditions. The dimeric form of CTLA-2β has also been reported, but this was a non-covalent complex of the monomer (Delaria et al.; 1994). The dimeric form of CTLA-2α, converted to the monomeric form by treatment with DTT, completely lost its inhibitory activity. DTT is known to be a strong reducing reagent that reduces disulfides to dithiols and then maintains these thiols in a reduced state. Therefore, our results indicate that the CTLA-2α monomer with a free thiol residue was not inhibitory. By removing DTT from the buffer, part of the monomer reassociated to form a dimer, which suggests that the monomer and dimer forms of CTLA-2α exist in dynamic equilibrium in solution. Interestingly, the inhibitory potency of the CTLA-2α monomer recovered to the level of the dimer by oxidization with glutathione disulfide (GSSG). These results imply that the cysteine residue of

CTLA-2α (C75), acting as a disulfide form, engaged in the inhibitory process. This result is in accordance with previous findings in which the cysteine residue (C75) was shown to be one of the essential amino acids of CTLA-2α for its inhibitory potency (Deshapriya et al.; 2010). Moreover, present attempts to isolate the CTLA-2α/cathepsin L complex have revealed that, in the process of inhibition, CTLA-2α may be covalently bound to the catalytic subunit of cathepsin L via the cysteine residue (C75). The catalytic cysteine residue (C25E) of cathepsin L is located close to the cysteine residue of CTLA-2α (C75), as has been suggested by molecular modeling analysis of the CTLA-2α/cathepsin L complex (Deshapriya et al.; 2010). CTLA-2α may inhibit cathepsin L-like cysteine protease by oxidizing the active thiol residue of the enzyme with its own thiol residue. Successful isolation of CTLA-2α/cathepsin L complex with a disulfide bond from the pregnant mouse uterus, suggesting this may occur in vivo. This result suggested that *in vivo* CTLA-2α may inhibit cathepsin L-like cysteine protease by oxidizing the active thiol residue of the enzyme with its own thiol residue.

Interaction between CTLA-2α and cathepsin C

Cathepsin C is a lysosomal cysteine protease that sequentially removes dipeptides from the unsubstituted N-terminus of target proteins with broad specificity (Turk et al.; 1968). This study demonstrated for the first time that CTLA-2α interacted with and is a potential endogenous inhibitor of cathepsin C. The isolation of the CTLA-2α /cathepsin C complex from pregnant mouse uteri signifies the occurrence of the interaction in physiogical conditions.

The simple cell-based fluorescence assay also revealed that the GF-AFC

substrate entered intact cells and was cleaved by cathepsin C to release AFC, leading to an increase in fluorescence intensity. The presence of CTLA-2α led to a significant reduction in the intensity of labeling in cathepsin C-expressing cells. This suggests that CTLA-2α may suppress cathepsin C activity *in vivo*. Inhibitory activity was not observed *in vitro* using purified cathepsin C, similar to cystatin F, a cathepsin C-directed protease inhibitor. Cystatin F was previously shown to be unable to inhibit cathepsin C *in vitro (*Langerholc et al.; 2005*)*, but suppressed the activity of endogenous cathepsin C *in vivo (*Hamilton et al.; 2008*)*. We also attempted to analyze the mechanism underlying this interaction. Our results suggested that CTLA-2α interacted with cathepsin C via a disulfide bond, similar to the interaction with cathepsin L.

Interaction between CTLA-2α and TINAGL1

Although TINAGL1 is not a peptidase, it has been identified as a protein homologous to cysteine proteases such as cathepsin L, B, and C (http://merops.sanger.ac.uk). The C-terminal of TINAGL1 was shown to be homologous to cathepsin B, but the active cysteine residue was replaced with a serine residue, which resulted in a proteolytically inactive protein (Bromme et al.; 2000). The structure and biological functions of this protein are still not well characterized. TINAGL1 is a matricellular protein that interacts with both structural proteins and cell surface receptors (Bromme et al.;, 2000). Using the yeast system, a strong interaction was observed between CTLA-2α and TINAGL1. However, it was not possible to isolate the CTLA-2α/TINAGL1 complex from mouse tissue by Co-IP. This was due to molecular weight of TINAGL1 being very close to immunoglobulin G (IgG; ~53kDa), and the difficulties associated with distinguishing between the target

protein (TINAGL1) and IgG leaking from the beads. TINAGL1 is a secretory protein (Wex et al.; 2001). Similarly, as described above part of CTLA-2α is secreted out of cells. Thus, pull-down assay was used to isolate the CTLA-2α/TINAGL1 complex from the culture medium of TINAGL1-expressing cells suggesting that the interaction between CTLA-2α and TINAGL1 occurs *in vivo*.

Location of CTLA-2α in the placenta

A previous study detected the expression of the CTLA-2α gene in the uterus during late pregnancy (Campo et al.; 1996). However, the tissue locations and physiological functions of CTLA-2α in the mouse placenta remain unclear. Immunofluorescence showed that CTLA-2α was primarily localized on the maternal side including the DB, MG, and MM layers, but was not observed on the embryonic side at different developmental stages from day 8 to day 17. Its isoform, the CTLA-2β protein, was also reported to be expressed in the pregnant uterus (Campo et al.; 1996). Thus, the cross-reactivity can happen between anti- CTLA-2α Ab and CTLA-2β. However, the expression of CTLA-2β in pregnant mouse uterus was restricted between day 4 to day 9 but was not observed on day 10. These findings indicate that from day 10, immunoreactivity specific detected CTLA-2α protein in the mouse placenta.

Besides, trophoblast-specific protein (TPBP), which is expressed in the placenta, is homologous to CTLA-2α. Thus, the cross-reactivity can happen between anti- CTLA-2α Ab and TPBP protein. However, TPBP is known to be located only in the spongiotrophoblast layer (Deussing et al.; 2013) and the immunoreactivity of CTLA-2α was seen consistently in the maternal side not in the spongiotrophoblast

layer

The expression of the three CTLA-2α interactive proteins, cathepsin L, cathepsin C, and TINAGL1, has been reported previously in the placenta (Igarashi et al.; 2009; Hamilton et al.; 1991; Menkhost et al.; 2012). The results of the present study revealed the different expression profiles of cathepsin L, cathepsin C, and TINAGL1 in the mouse placenta. Results of this study also clearly demonstrate co-localization of CTLA-2α in some regions with cathepsin L, cathepsin C, and TINAGL1.

Co-localization of CTLA-2α and cathepsin L in the placenta

Cathepsin L is shown to be present in large amounts in the placenta, implying that Cathepsin L plays important role in the placenta (Hamilton et al.; 1991). The present study also shows that cathepsin L is ubiquitously expressed in the placenta and co-localizes with CTLA-2α in the DB and MG regions. Cathepsin L is important for the proteolytic processing of extracellular matrices, cellular invasion and migration (Hamilton et al.;1991; Krischke et al.; 2012) and a part of it has also been reported to be secreted in the placenta (Hamilton et al.;1991), and regulation of trophoblast invasion is an important process for placental formation. Taking these data altogether, it is stipulated that CTLA-2α, which inhibits cathepsin L activity, may be involved in the decidualization reaction and normal placental formation. Placenta-specific cysteine peptidases, which are referred to as cathepsin L-related proteins (CLRPs) due to their high degree of similarity to cathepsin L (Deusing et al.; 2009), can be inhibited by CTLA-2α. CTLA-2α was consistently expressed on the maternal side, whereas CLPRs was detected on the embryonic side such as the labyrinth and spongiotrophoblast (Deusing et al.; 2009). This implies that CTLA-2α does not control the activities of CLRPs.

Co-localization of CTLA-2α and cathepsin C in the placenta

The expression profile of cathepsin C in the placenta differed from that of cathepsin L. Cathepsin C was previously shown to be weakly expressed in the placenta (Menkhost et al.; 2012). In this study, the co-expression signal of cathepsin C and CTLA-2α was detected in the DB and MG. CTLA-2α may regulate cathepsin C activity as an inhibitor in these regions. Cathepsin C plays an important role in various physiological and pathological progresses (Turk et al.; 2012) and it is required for the activation of serine proteases from granules of immune and inflammatory cells. Granzymes A and B, two targets of cathepsin C, are important factors that mediate apoptotic pathways (Turk et al.; 2012; Pham CTN et al.; 1999). In addition to the placenta, CTLA-2α was detected in the brain and eyes (Luziga et al.; 2007, 2008). All these organs have been identified as immune-privileged sites, in which immune-tolerance mechanisms that operate to protect tissues from immune-mediated damage are known to be established (Mochizuki et al.; 2010). In the developing placenta, abundant numbers of "uterine natural killer cells" are existed at the regions of DB and MG. These cells possess granzyme B and have a possibility to secrete against the fetal tissue (Parr et al.; 1990). Taken together, these findings suggest that CTLA-2α, through its inhibitory activity towards cathepsin C, may be involved in the processes that protect the embryo from the maternal immune system.

Co-localization of CTLA-2α and TINAGL1 in the placenta

The expression of TINAGL1 in the decidua was previously demonstrated to support post-implantation embryonic development (Tajiri et al.; 2010). Furthermore, TINAGL1 has been shown to promote endothelial cell adhesion (Li et al.; 2007), and

is essential to angiogenesis in developing zebrafish embryos (Brown, 2010). Since high levels of TINAGL1 was detected in the placenta from day 6 to day 8 (Li et al.; 2007), immunohistochemical studies were performed in 8 dpc uteri to examine the co-localization of CTLA-2α and TINAGL1. The distribution profiles of CTLA-2α and TINAGL1 were similar in 8 dpc-mouse uteri. Therefore, we hypothesized that CTLA-2α, with the interaction of TINAGL1, may be involved in blocking cell invasion and migration during early post-implantation periods.

Conclusion

This is the first study to provide direct evidence for the interaction between CTLA-2α and cathepsin L *in vivo*. Novel interactions between CTLA-2α and cathepsin C and TINAGL1 were also demonstrated. Results of this study suggest that CTLA-2α mediates Cathepsin L, Cathepsin C and TINAGL1 *in vivo*. Their interactions in the placenta suggest important function of these proteins in embryo implantation and development. More research is needed to help better understand the role of CTLA-2α.

REFFERENCES

[1] Adkison, A.M., Raptis, S.Z., Kelley, D.G., Pham, C.T.N. Dipeptidyl peptidase I activates neutrophil-derived serine proteases and regulates the development of acute experimental arthritis. The J.Clin.Invest. 2010; 109: pp. 363-371.

[2] Barrett, A. J., Kirschke, H. “Cathepsin B, cathepsin H and cathepsin L.” In: Lorand. L, Editor. Methods in Enzymology. Academic Press, New York. 1981; vol 80: pp. 535-561.

[3] Barrett, A. J., Woessner. J.FJ. In: Rawlings. N.D. editor. Handbook of Proteolytic Enzymes. Academic Press, London. 1998; pp. 235-254

[4] Berti, P.J., Storer, A.C. Alignment/phylogeny of the papain superfamily of cysteine proteases. J. Mol. Biol. 1995; 246: pp. 273-283

[5] Bromme, N.C., Wex, T., Wex, H., Levy, B., Lipyansky, A., Bromme, D. Cloning, characterization, and expression of the human TINAGL1 gene encoding a novel extracellular matrix protein. Biochem. Bioph. Res. Co. 2000; 271:474-480.

[6] Brömme, D., Wilson, S. Role of cysteine cathepsins in extracellular proteolysis. In: Parks WC, Mecham RP, editors. Extracellular matrix degradation. Springer-Verlag; 2011; pp. 23-44.

[7] Brown, L.J., Alawoki, M., Crawford, M.E., Reida, T., Sears, A. Lipocalin-7 Is a Matricellular Regulator of Angiogenesis. PLoS ONE. 2010; 5(11): 1-11.

[8] Bui, T.T.N., Takeshita, Y., Yamamoto, M., Yamamoto, Y. Studies of inhibitory mechanisms of propeptide-like cysteine protease inhibitors. Enzyme res. 2014.

[9] Bui, T.T.N., Luziga, C., Kusakabe, K.T., Yamamoto, M., Yamamoto, Y. Identification and characterization of the interactive proteins with Cytotoxic T-lymphocyte antigen-2α. Biosci. Biotechnol. Biochem. 2014

[10] Camenisch, G., Tini, M., Chilov, D., Kvietikove, I., Srinivas, V., Caro, J., Spielmann, P., Wenger, R.H. General applicability of chicken egg yolk antibodies: the performance of IgY immunoglobulins raised against the hypoxia-inducible factor 1α. FASEB J. 1999; 13(1): pp. 13-81.

[11] Campo, M.A., Rice, E.J., Kasik, J.W. There is an increase in expression of the cytotoxic T-lymphocyte antigen-2α gene during pregnancy. Am. J. Obstet. Gynecol. 1996; 174(5): pp. 1605-1607.

[12] Carmona, E., Dufour, E., Plouffe, C., Takebe, S., Mason, P., Mort, J.S., Menard, R. Potency and selectivity of the cathepsin L propeptide as an inhibitor of cysteine proteases. Biochemistry. 1996; 35: pp. 8149-8157.

[13] Cheon, Y.P., DeMayo, F.J., Bagchi, M.K., Bagchi, I.C. Induction of cytotoxic T-lymphocyte antigen-2β, a cysteine protease inhibitor in decidua: a potential regulator of embryo implantation. J. Biol. Chem. 2004; 279(11): pp. 10357-10363.

[14] Chen, Y., Plouffe, C., Menard, R., Storer, A.C. Delineating functionally important regions and residues in the cathepsin B propeptide for inhibitory activity. FEBS Lett. 1996; 39: pp. 24-26.

[15] Chowdhury, S.F., Sivaraman, J., Wang, J., Devanathan, G., Lachance, P., Qi, H., Ménard, R., Lefebvre, J., Konishi, Y., Cygler, M., Sulea, T., Purisima, E.O. Design of noncovalent inhibitors of human cathepsin L. from the 96-residue proregion to optimized tripeptides. Journal of Medical Chemistry. 2002; 45: pp. 5321-5329.

[16] Chien, C.T., Bartel, P.L., Sternlanz, R., Fields, S. Proc. Natl. Acad. Sci. USA. 1991; 88: pp. 9578-9582.

[17] Coulombe, R. P., Grochuiski, J., Sivarman, R., Ménard, J., Mort, S.,Cygler, M. Structure of human procathepsin L reveals the molecular basis of inhibition by the prosegment. EMBO Journal 1996; 15(20): pp. 5492-550.

[18] Cygler, M., Mort, J.S. Proregion structure of members of the papain superfamily. Mode of inhibition of enzymatic activity. Biochimie. 1997; 79: pp. 645-652

[19] Delaria, K., Fiorentino, L., Wallace, L., Tamburini, P., Brownell, E., Muller, D. Inhibition of cathepsin L-like cysteine proteases by cytotoxic T-lymphocyte antigen-2β. Biol. Chem. 1994; 269: pp. 25172-25177.

[20] Denizot, V., Brunet, J.F., Roustan, P., Harper, K., Suzan, M., Luciani, M.F., Mattei, M.G.P., Golstein, P. Novel structures CTLA-2α and CTLA-2β expressed in mouse activated T cells and mast cells and homologous to cysteine proteinase proregions. Eur. J. Immunol. 1989; 19: pp. 631-635.

[21] Deshapriya. R.M.C., Yuhashi, S., Usui, M., Kageyama, T., Yamamoto, Y. Identification of essential residues of CTLA-2α for inhibitory potency. J. Biochem. 2010; 147(3): pp. 393-404.

[22] Deshapriya, R. M. C., Takeuchi, A., Shirao, K., Isa, K., Watabe, S., Murakami, M., Tsujimura, H., Yamamoto, Y. Drosophila CTLA-2-like protein (D/CTLA-2) inhibits cysteine proteinase 1 (CP1), a cathepsin L-like enzyme. Zoological Science. 2007; 24(1): pp. 21-30.

[23] Deussing, J. Placenta-specific Cathepsins. In: Barrett AJ, Rawlings ND, Woessner JF, editors. Handbook of Proteolytic Enzymes. Volume.1. 2nd edition. Elsevier Academic Press; 2012. p. 1945-1851.

[24] Deussing, J., Koudio, M., Rehman, M., Werber, I., Schwinde, A., Peter, C. Identification and characterization of a dense cluster of placenta-specific cysteine peptidase genes and related genes on mouse chromosome 13. Genomics. 2012; 79: pp. 225-240.

[25] Guo, Y.L., Kurz, U., Schultz, J.E., Lim, C.C., Wiederanders, B., Schilling, K. The a1/2 helical backbone of the prodomains define the intrinsic inhibitory specificity in the cathepsin L- like cysteine protease subfamily. FEBS Lett. 2000; 469: pp. 203-207.

[26] Fabien, L., Brömme, D. Human and Parasitic Papain-Like Cysteine Proteases: Their Role in Physiology and Pathology and Recent Developments in Inhibitor Design. *Chem. Rev.* 2002; *102* (12): pp: 4459–4488.

[27] Fields. S., Song.O. Nature. 1989; 340: pp. 245-246.

[28] Hamilton, G., Colbert, J., Schuettelkopt, A., Watts, C. Cystatin F is a Cathepsin C-directed protease inhibitor regulated by proteolysis. The EMBO J. 2008; 27: pp. 499-508.

[29] Hamilton, R., Bruns, K., Delgado, M., Shim, J., Fang, Y., Denhardt, D., Nilsen-Hamilton M. Developmental expression of Cathepsin L and c-ras^{Ha} in the mouse placenta. Mol. Reprod. Dev. 1991; 30: pp. 285-292.

[30] Igarashi,T., Tajiri, Y., Sakurai, M., Sato, E., Li, D., Mukai, K., Suematsu, M., Fukui, E., Yoshizawa, M., Matsumoto, H. Tubulointerstitial nephritis antigen-like 1 is expressed in extraembryonic tissue and interacts with Laminin 1 in the Reichert Membrane at postimplantation in the Mouse. Biol. Reprod. 2009; 80: pp. 948-955.

[31] Irischke, H. Cathepsin L. In: Barrett AJ, Rawlings ND, Woessner JF, editors. Handbook of Proteolytic Enzymes. Volume.1. 2nd edition. Elsevier Academic Press; 2012. pp. 1808-1817.

[32] Katunuma, N., Kominami, N. Structure, properties, mechanisms, and assays of cysteine protease inhibitors: cystatins and E-64 derivatives. In: Methods in Enzymology vol. 251, L. Packer, Ed., Academic Press, San Diego, 1995. pp. 382-397

[33] Kurata, M., Hirata, M., Watabe, D., Miyake, M., Takahashi, S.Y., Yamamoto, Y. Expression, purification, and inhibitory activities of mouse cytotoxic T-lymphocyte antigen-2α. Protein Expr. Purif. 2003; 32: pp. 119-125.

[34] Kurata, M., Yamamoto, Y., Watabe, S., Makino, Y., Ogawa, K., and Takahashi, S.Y. Bombyx cysteine proteinase inhibitor (BCPI) homologous to propeptide regions of cysteine proteinases is a strong, selective inhibitor of cathepsin L-like cysteine proteinases. J. Biochem. 2001; 130: pp. 857-863.

[35] Langerholc, T., Zavasnik-Bergant, V., Turk, V., Abrahamson, M., Kos, J. Inhibitory properties of cystation F and its colocalization in U937 promonocyte cells. FEBS J. 2005; 271: pp. 1535-1545.

[36] Lecaille, F., Kaleta, J., and Bromme, D. Human and parasitic-like cysteine proteases: their role in physiology and pathology and recent development in inhibitor design. Chem. Rev. 2002; 102: pp. 4459-4488.

[37] Li, D., Mukai, K., Suzuki, T., Suzuki, R., Yamashita, S., Mitani, F., Suematsu, M. Adrenocortical zonation factor 1 is a novel matricellular protein promoting integrin-mediated adhesion of adrenocortical and vascular smooth muscle cells. FEBS J. 2007; 274: pp. 2506–2522.

[38] Luziga, C., Nakamura, O., Deshapriya, R.M.C., Usui, M., Miyaji, M., Wakimoto, M., Wada, N., Yamamoto, Y. Expression mapping of cytotoxic T-lymphocyte antigen-2 alpha gene transcripts in mouse brain. Histochem. Cell Biol. 2007; 127(6): pp. 569-579.

[39] Luziga, C., Nakamura, O., Deshapriya, R.M.C., Usui, M., Miyaji, M., Wakimoto, M., Wada, N., Mbassa, G., Yamamoto, Y. Dendritic and axonal localization of cytotoxic T-lymphocyte antigen-2 alpha protein in mouse brain. Brain Res. 2008; 1204: pp. 40-52.

[40] Menkhost, E.M., Lane, N., Winship, A.L., Li, P., Yap, J. Decidual- secreted factors alter invasive trophoblast membrane and secreted protein implying a role for decidua cell regulation of placentation. PLoS ONE. 2012; 7(2): pp. 1-11.

[41] Methot, N., Rubin, J., Guay, D., Beaulieu, C., Ethier, D. Inhibition of the activation of multiple Serin protease with Cathepsin C inhibitor requires sustained exposure to prevent pro-enzyme precessing. J. Biochem. 2007; 282: pp. 20836-20846.

[42] Mochizuki, M. Regional immunity of the eye. Acta Ophthalmol. 2010; 88: pp. 292-299.

[43] Parr, E.L., Young, L.H., Parr, M.B., Young, J.D. Granulated metrial gland cells of pregnant mouse uterus are natural killer-like cells that contain perforin and serine esterases. J. Immunol. 1990; 145(7): pp. 2365-2372.

[44] Pham, C.T.N., Let, T.J. Dipeptidyl peptidase I is required for the processing and activation of granzymes A and B *in vivo*. Proceeding of National Academy of Sciences; 1999 June 20; USA. 96(15): pp. 8627-8632.

[45] Reddy, V.Y., Zhang, Q.Y., Weiss, S.J. Pericellular mobilization of the tissue-destructive cysteine proteinases, cathepsins B, L, and S, by human monocyte-derived macrophages. Proc. Natl. Acad. Sci. 1995; 92: pp3849–3853.

[47] Santilman, V., Jadot, M., Mainderme, F. Importance of the propeptide in the biosynthetic maturation of rat Cathepsin C. Eur. J. Cell Biol. 2002; 81: pp. 654-663.

[48] Sugita, S., Yamada, Y., Horie, S., Nakamura, O., Futagami, Y., Yamamoto, Y., Yamagami, S., Mochizuki, M. Induction of T Regulatory Cells by Cytotoxic T-Lymphocyte Antigen-2α on Corneal Endothelial Cells. Invest. Ophthalmol. Vis. Sci. 2011; 52: pp. 2598-2605.

[49] Sugita. S., Horie, S., Nakamura, O., Futagami, Y., Takase, H., Keino,H., Aburatani, H., Katunuma, N., Ishidoh, K., Yamamoto, Y., Mochizuki, M. Retinal pigment epithelium derived cytotoxic T lymphocyte antigen-2α induces TGFβ-producing T regulatory cells. J. Immunol. 2008; 181: pp. 7525-7536.

[50] Sugita, S., Horie, S., Nakamura, O., Maruyama, K., Takase, H., Usui, Y., Takeuchi, M., Ishidoh, K., Koike, M., Uchiyama, Y., Peters, C., Yamamoto, Y., Mochizuki, M. Acquisition of T regulatory function in cathepsin L-inhibited T cells by eye-derived CTLA-2 during inflammatory conditions. J. Immunol. 2009; 183: pp. 5013 – 5022.

[51] Rao, N.V., Rao, G.V., Hoidal, J.R. Human Dipeptidyl peptidase I. J. Bichem. 1997; 272: pp. 10260-10265.

[52] Rawlings, N.D., Barrett, A.J. Families of Cysteine peptidases. Meth. Enzymol. 1994; 244: pp. 461-486.

[53] Takahashi, S., Zhao, X., Kageyama, T., Yamamoto, Y. Acid cysteine proteinase from the eggs of silkmoth, Bombyx mori: Tissue distribution

developmental changes and the sites of synthesis for the enzyme. Insect Biochem. Molec. 1992; 22(4): pp. 369-377.

[54] Tao, K., Stearns, N.A., Dong, J., Wu, Q.-L., and Sahagian, G.G. The proregion of cathepsin L is required for proper folding, stability and ER exit. Arch.Biochem. Biophys. 1994; 311: pp. 19-27.

[55] Tajiri, Y., Igarashi, T., Li, D., Mukai, K., Suematsu, M., Fukui, F., Yoshizawa, M., Matsumoto, H. Tubulointerstitial Nephritis Antigen-Like 1 Is Expressed in the Uterus and Binds with Integrins in Decidualized Endometrium During Postimplantation in Mice. Biol. Reprod. 2010; 82: pp. 263-270.

[56] Thong,B., Pilling, J., Ainscow, Beri., Unitt, J. Development and validation of a simple Cell-based fluorescence assay for Dipeptidyl Peptidase 1 (DPP1) activity. J. Biomol. Screen. 2011; 16(1): pp. 36-43.

[57] Turk, B., Turk, D., Dolenc, I., Turk, V. Dipeptidyl-Peptidase I. In: Barrett AJ, Rawlings ND, Woessner JF, editors. Handbook of Proteolytic Enzymes. Volume.1. 2nd edition. Elsevier Academic Press; 2012. pp. 1968-1973.

[58] Turk, B., Dolenc, I., Turk, V., and Bieth, J.G. Kinetics of the pH induced activation of human cathepsin L. Biochemistry. 1993; 32: pp. 375-382.

[59] Turk, V., Stoka, V., Vasiljeva, O., Renko, M., Sun, T., Turk, B., Turk, D. Cysteine cathepsins: from structure, function and regulation to new frontiers. Biochimica et Biophysica Acta. 2012; 1824: pp. 68-88

[60] Wex, T., Lipyansky, A., Bromme, N.C., Wex, H., Guan, X.Q., Bromme, D. TINAGL1, a novel catalytically inactive cathepsin B-related protein with EGFdomains, is predominantly expressed in vascular smooth muscle cells. Biochemistry. 2001; 40: pp. 1350–1357.

[61] Wiederanders, B., Kaulmann, G., and Schilling, K. Function of propeptide parts in cysteine proteases Curr. Protein Pept. Sci. 2003; 4: pp. 309-326.

[62] Wiederanders, B. Structure-function relationship in class CA1 cysteine peptidase propeptides. Acta Biochim. Pol. 2003; 50: pp. 691-713.

[63] Wakamatsu, N., Kominami, E., Takio, K., Katunuma, N. Three forms of thiol proteinase inhibitor from rat liver formed depending on the oxidation-reduction state of a sulfhydryl group. Journal of Biological Chemistry. 1984; 259(22): pp. 13832-13838.

[64] Yamamoto, Y., Kurata, M., Watabe, S., Murakami, R., and Takahashi, S.Y. Novel cysteine proteinase inhibitors homologous to the proregions of cysteine proteinases. Curr. Protein Pept. Sci. 2002; 3: pp. 231-238

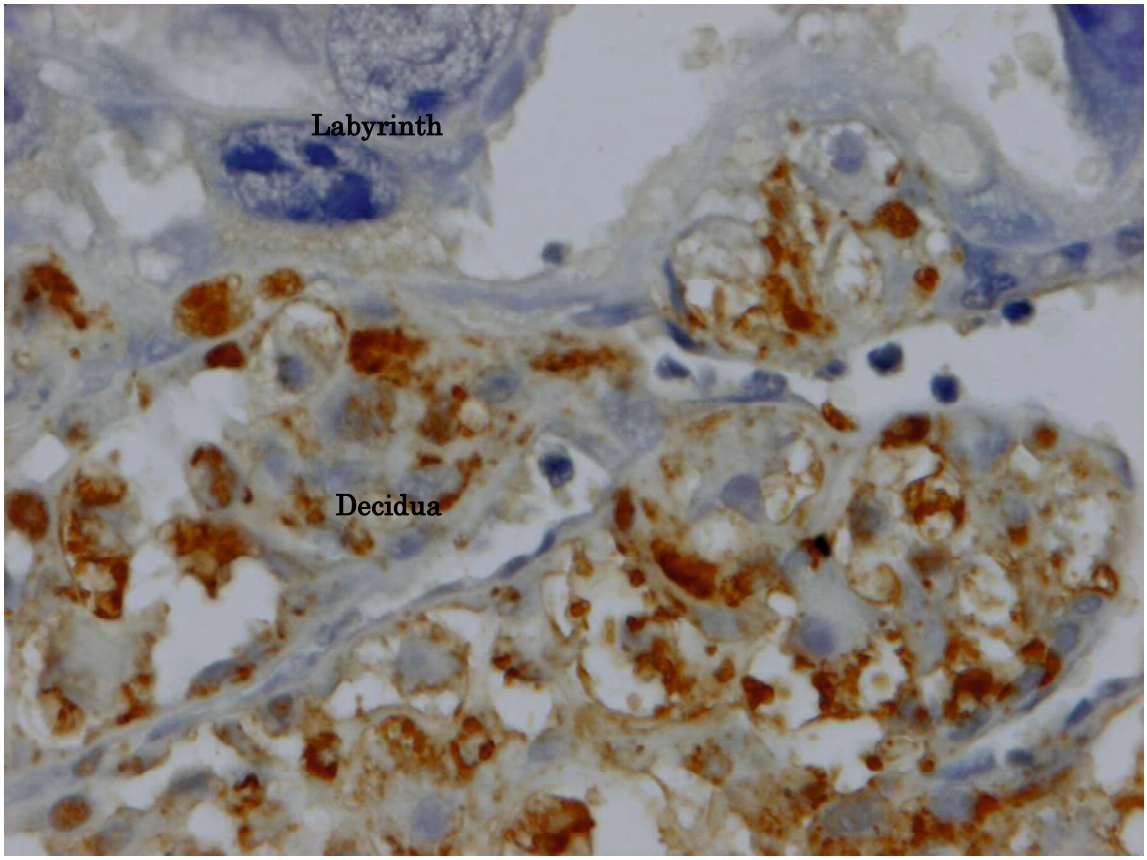
[65] Yamamoto, Y., Watabe, S., Kageyama, T., and Takahashi, S.Y. Purification and characterization of Bombyx cysteine proteinase specific inhibitors from the hemolymph of Bombyx mori. Arch. Insect Biochem. Physiol. 1999; 41: pp. 119-129

[66] Yamamoto, Y., Watabe, S., Kageyama, T., and Takahashi, S.Y. A Novel inhibitor protein for Bombyx cysteine proteinase is homolgous to propeptide regions of cysteine proteinases. FEBS Lett. 1999; 448: pp. 257-260

[67] Zhang, L., Yun, H., Murray, F., Lu, R., Wang, L., Hook, V. Insel, P.A. Cytotoxic T lymphocyte antigen-2 alpha induces apoptosis of murine T-lymphoma cells and cardiac fibroblasts and is regulated by cAMP/PKA. Cellular Signal. 2011; 23(10): pp. 1611-1616.

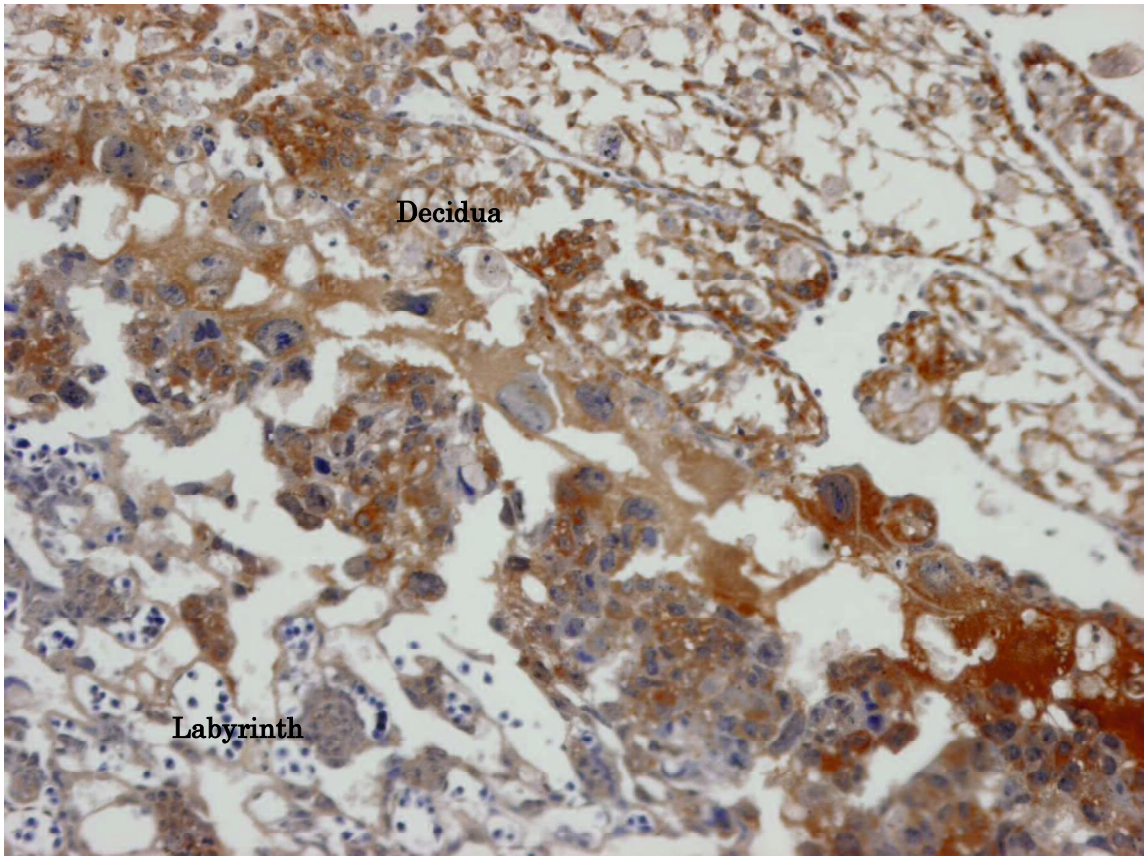
APPENDICES

Appendix I: Localizaion of CTLA-2α in the mouse placenta.



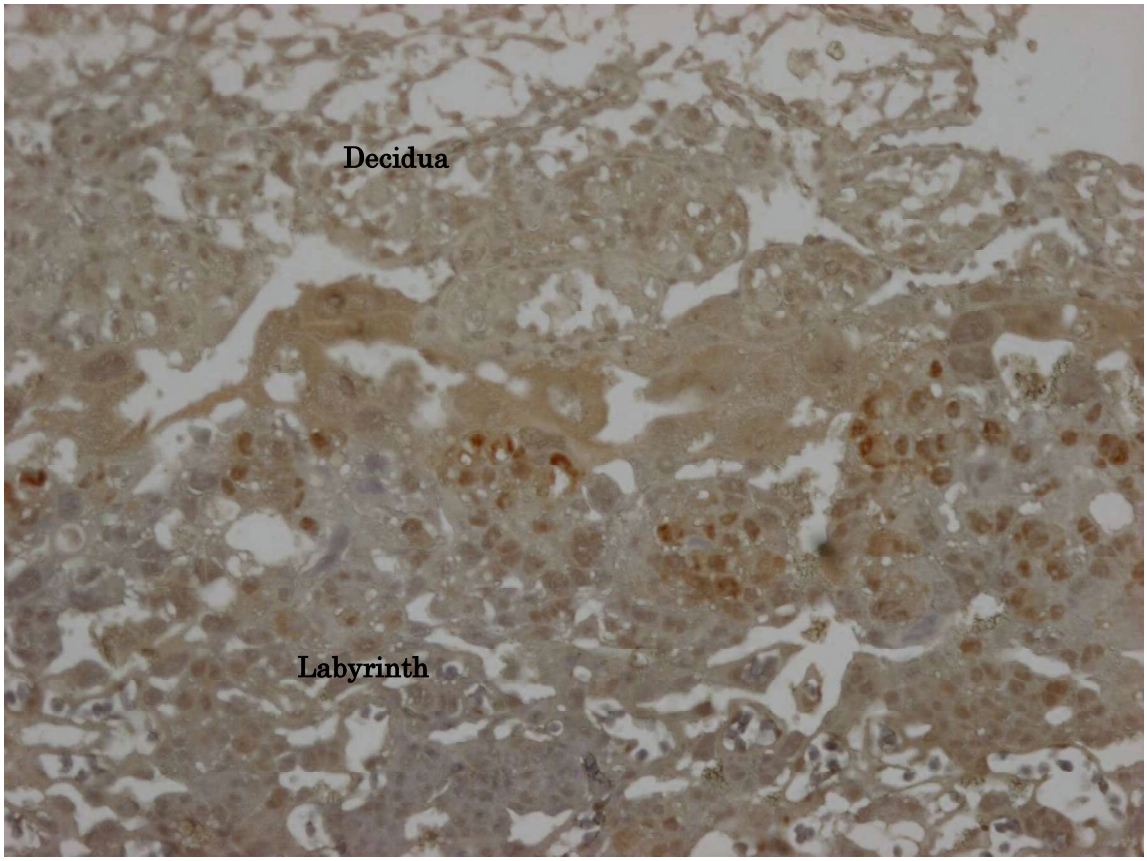
Appendix II:

Localization of cathepsin L in the mouse placenta



Appendix III:

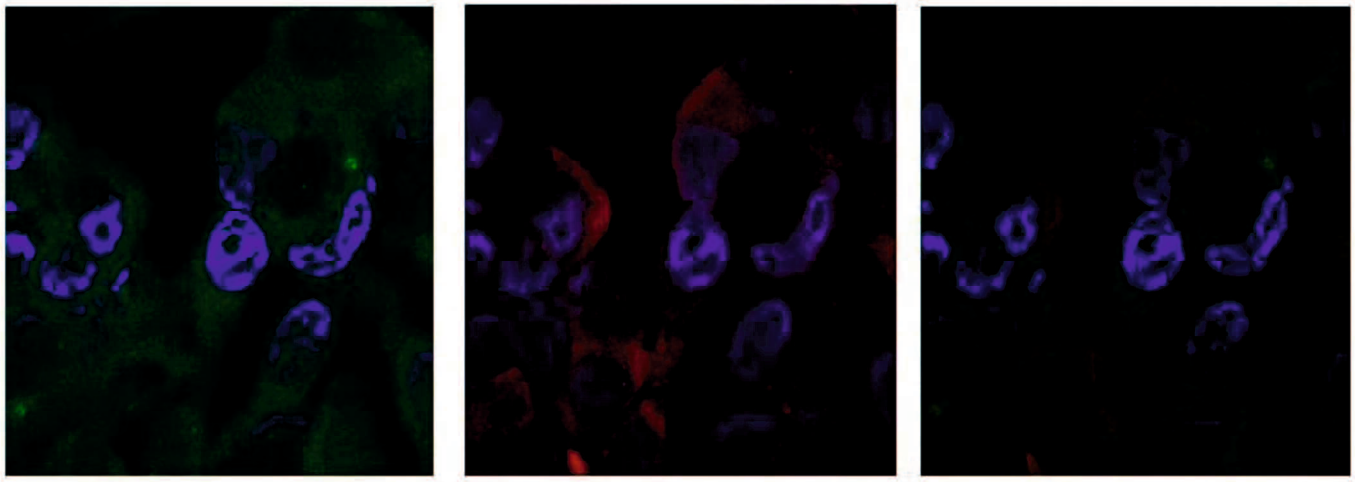
Localization of cathepsin C in the mouse placenta



Appendix IV: Detection the localization of CTLA-2α by confocal system.

Double immuno-staining using anti- CTLA-2α and anti-LAMP1 (lysosome marker) Abs have been performed on HEK293T cells transfeted with CTLA-2α. As can be seen in the figure appendix 4, CTLA-2α (red) presented with lysosomes (green).

A B Overlay image



Appendix V: Detection the regulatory T cells in the mouse placenta.

Previous studie shows that CTLA-2α is involved in the formation of regional immunity in the eye. Retinal pigment epithelium-derived CTLA-2α has the ability to generate T reg cells. Therefore, immunohistochemistry with anti-FOXP3 (T reg cells marker) Ab has been performed to detect the Treg cells in placenta. At the stage 10 dpc, T reg presented in decidua layer with small population compare to labyrith layer.