Functional Analysis of an Apoptosis-related Molecule, Programmed Cell Death 5 of *Toxoplasma gondii*

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Contents

Abbreviations

A aa: Amino acids

AIDS: Acquired immunodeficiency syndrome

ARP: Apoptosis-related protein

B BSA: Bovine serum albumin

BLAST: Basic local alignment search tool

C CTL: Cytotoxic T cell

D dsDNA: Double-strand DNA

E ELISA: Enzyme-linked immunosorbent assay

EST: Expressed sequence tag

ESP: Excretory/secretory protein

G GFP: Green fluorescent protein

GST: Glutathione *S*-transferase

H HRP: Horseradish peroxidase

HSPG: Heparan sulfate proteoglycan

I IAP: Inhibitors of apoptosis proteins

IEM: Immuno-electron microscopy

IFAT: Indirect fluorescence antibody test

IFN-γ: Interferon-gamma

IκBα: Inhibitor-kappa B-alpha

IKK: Inhibitor-kappa B kinase

IL: Interleukine

IgG: Immunoglobulin G

N NCBI: National Center for Biotechnology Information

NF-κB: Nuclear factor-kappa B

NK: Natural killer

NO: Nitric oxide

P PBS: Phospate-buffered saline

PCR: Polymerase chain reaction

PDCD5: Programmed Cell Death 5

PFA: Paraformaldehyde

PI: Propidium iodide

PV: Parasitophorous vacuole

R r: Recombinant

S SDS-PAGE: Sodium dodecyl sulfate polyacrylamide gel electrophoresis

T TGF-β: Transforming growth factor-beta

TNF-α: Tumor necrosis factor-alpha

TUNEL: Terminal deoxynucleotidyltransferase-mediated dUTP-biotin nick-end labeling

General introduction

1. The importance of *Toxoplasma* parasite

Toxoplasma gondii is an obligate intracellular parasite assigned to the phylum Apicomplexa and class Sporozoa [Beverly, 1976]. It infects virtually all species of warm-blooded animals, including humans, worldwide. Domestic cats and other felines are the definitive hosts, and all non-feline hosts are intermediate hosts.

T. gondii infection is usually asymptomatic in immunocompetent host, and only rare severe cases occur. However, it is still a serious threat to human health due to its high prevalence, which is typically 10-30% in most populations, or even higher. For example, it is the third most common cause of food-borne deaths in the United States [Mead et al., 1999]; furthermore, several cases of ocular symptoms, such as retinitis and retinochoroiditis, were reported as acquired toxoplasmosis due to several outbreaks of acute toxoplasmosis [Masur et al., 1978; Bowie et al., 1997; Choi et al., 1997; Burnett et al., 1998]. Vertical transmission of *T. gondii* from mother to fetus during gestation may cause congenital toxoplasmosis, characterized clinically by abortion, neonatal death, or fetal abnormalities with detrimental consequence for the fetus, especially when the transmission occurs at an early stage of pregnancy [Dubey and Beattie, 1988; Tenter et al., 2000]. It may also significantly reduce the quality of life in children who survive a prenatal infection [Roberts et al., 1994; Foulon et al., 1999; Tenter et al., 2000]. Recent estimates based on serological studies suggested incidences of primary maternal infection during pregnancy to range from about 1-310 per 10,000 pregnancies in different population in Europe, Asia, Australia, and the Americas [Tenter et al., 2000]. About 10% of the prenatal infections result in abortion

or neonatal death. Another 10-23% of prenatal infected newborns show clinical signs of toxoplasmosis at birth [Tenter et al., 2000]. In immunocompromised humans, a previously acquired latent infection can lead to reactivated toxoplasmosis with encephalitis. Toxoplasmic encephalitis and disseminated toxoplasmosis have been observed in patients with immunodediciencies due to various causes, such as Hodgkin's disease or immunosuppressive therapy because of other malignancies. Disseminated toxoplasmosis may also complicate transplantation of organs or bone marrow. *T. gondii* has emerged as one of the most common opportunistic infections in patients with the acquired immunodeficiency syndrome (AIDS) [Wanke et al., 1987]. It causes severe encephalitis in up to 40% of AIDS patients worldwide, and 10-30% of AIDS patients infected with *T. gondii* succumb to the disease [Tenter et al., 2000].

On the other hand, toxoplasmosis is one of the main causes of infectious reproductive wastage in sheep, and goats. It may cause fetal resorption, abortion, stillbirth, and neonatal mortality [Buxton et al., 1998; Freyre et al., 1999; Duncanson et al., 2001]. Although toxoplasmosis in pigs is not severe production problem, clinical cases has been occurred in young pigs [Dubey, 1986]. In addition to the problem of such clinical toxoplasmosis in animals, tissue cysts of *T. gondii* contained in meat of livestock are an important source of infection for humans. In meat-producing animals, tissue cysts of *T. gondii* are most frequently observed in tissues of infected pigs, sheep, and goats, and less frequently in infected poultry, rabbits, dogs, and horses [Dubey, 2000; Tenter et al., 2000]. Therefore, *T. gondii* is of great importance in medical, veterinary medical, and food hygiene aspects.

2. The life cycle of *T. gondii*

There are three infectious stages: sporozoites in oocysts, invasive tachyzoites (actively multiplying stage), and encysted bradyzoites (slowly multiplying stage) [Dubey and Beattie, 1988]. Oocysts are excreted in feces, whereas tachyzoites and bradyzoites are found in tissues. Three major ways of transmission are congenital infection, ingestion of infected tissues, and ingestion of oocyst-contaminated food or water. Other minor modes of transmission include infected milk and transfusion of fluids or transplantation of organs [Dubey and Beattie, 1988; Jackson and Hutchison, 1989; Remington and Desmonts, 1990; Evans, 1992; Dubey, 1993; Johnson, 1997].

The enteroepithelial life cycle is found only in the definitive feline host. Most cats become infected by ingesting intermediate hosts infected with tissue cysts. Bradyzoites are released from the tissue cysts in the stomack and intestine when the cyst wall is dissolved by digestive enzymes. Bradyzoites penetrate the epithelial cells of small intestine and initiate the asexual stages. After an undetermined number of generations, merozoites form male (micro) and female (macro) gamonts. The microgamont divides and forms several biflagellate microgametes, which are released and swim to and penetrate macrogamonts. A wall is formed around the fertilized macrogamont to form an oocyst. After exposure to air and moisture, oocysts sporulate and contain two sporocysts, each with four sporozoites [Dubey and Beattie, 1988; Jackson and Hutchison, 1989; Evans, 1992; Dubey, 1993; Dubey et al., 1998]. The entire enteroepithelial cycle of *T. gondii* can be completed within 3 days after ingestion of tissue cysts. However, following ingestion of oocysts or tachyzoites, the formation of oocysts is delayed until 3 weeks or more.

The extraintestinal development of *T. gondii* is the same for all warm

blooded-hosts. After ingestion of oocysts, sporozoites excyst in the lumen of the small intestine and penetrate intestinal cells including the cells in the lamina propria. Sporozoites divide into two by an asexual process known as endodyogeny and become tachyzoites. They multiply in almost any kinds of cells in the body. If the cells rupture, they infect new cells [Dubey and Beattie, 1988; Jackson and Hutchison, 1989; Evans, 1992; Dubey, 1993; Dubey et al., 1998]. Otherwise, they multiply intracellularly for an undetermined period and eventually encyst. Tissue cysts grow intracellularly and contain numerous bradyzoites, frequently observed in the central nerve system, muscles, and visceral organs and probably persist for the life of the hosts [Dubey, 1993, 1998; Dubey et al., 1998].

3. Apoptosis inhibition in host cells by *T. gondii* infection

Apoptosis is a complex and highly regulated molecular pathway present in the cells of metazoans [Rich et al., 1999; Saraste and Pulkki, 2000]. In addition to its critical roles in development and tissue remodeling, apoptosis plays an important role in responding to infectious agents [Barcinski and DosReis, 1999; DosReis and Barcinski, 2001]. Apoptosis is often triggered in response to infection with viruses, bacteria, and protozoa [Roulston et al., 1999; Gao and AbuKwaik, 2000; Heussler et al., 2001]. Programmed suicide of infected cells soon after infection slows the growth of the pathogen resulting in the mitigation of infection.

T. gondii-mediated resistance against apoptosis was observed in both murine and human cell lines treated with diverse inducers of apoptosis, including cytotoxic T lymphocyte (CTL)-mediated cytotoxicity, irradiation, growth factor withdrawal, tumor necrosis factor (TNF)-α, and/or several toxic agents [Nash et al., 1998; Goebel et al.,

2001; Payne et al., 2003]. Decreased apoptosis in primary cells cultured *ex vivo* after growth factor withdrawal was also reported [Hisaeda et al., 1997; Channon et al., 2002]. Importantly, inhibition of apoptosis has recently also been shown to occur *in vivo* after intraperitoneal infection of mice with *T. gondii* [Orlowsky et al., 1999, 2002]. This suggests that interference of *T. gondii* with the suicide program of host cells may modify the course of toxoplasmosis. Mechanisms that may inhibit the mitochondrial apoptotic pathway in *T. gondii*-infected cells include: blocking mitochondrial cytochrome *c* release [Goebel et al., 2001; Carmen et al., 2006], altering the balance between pro- and anti-apoptotic Bcl-2 proteins [Goebel et al., 2001; Nishikawa et al., 2002; Molestina et al., 2003; Carmen et al., 2006], and direct inhibition of cytochrome *c*-mediated activation of the caspase cascade [Keller et al., 2006]. Inhibition of apoptosis by *T. gondii* may also be related to a parasite-driven increase of inhibitors of apoptosis proteins (IAP) which directly inhibits distinct caspases [Blader et al., 2001; Molestina et al., 2003; Lüder and Groß, 2005]. Parasite-derived inhibitor-kappa B kinase (IKK) was found to have the ability to phosphorylate inhibitor-kappa B-alpha (IκBα), which may result in translocation of nuclear factor-kappa B (NF-κB) dimers to the nucleus and up-regulation of IAP [Molestina et al., 2005].

4. Apoptosis induction in host cells by *T. gondii* infection

Acute infection of both humans and mice with *T. gondii* induces as state of transient immunosuppression as determined by decreased antibody and T lymphocyte responses to antigens [Strickland et al., 1977; Wing et al., 1983; Luft et al., 1984; Yano et al., 1987]. Among other factors, apoptosis of T lymphocytes triggered by *T. gondii* may restrict the immune response to the parasite [Khan et al., 1996; Liesenfeld et al.,

1997; Wei et al., 2002]. Indeed, high levels of apoptosis in splenocytes have been associated with unrestricted parasite multiplication leading to high parasite burdens in various tissues of the host [Gavrilescu and Denkers, 2001; Mordue et al., 2001]. Lymphocyte apoptosis may also influence the local immune response after natural parasite transmission via the gut, because oral infection with *T. gondii* led to apoptosis in Peyer's patch T cells [Liesenfeld et al., 1997]. Apoptosis was also observed in inflammatory cells and eye tissue after intraocular inoculation of *T. gondii* [Hu et al., 1999]. In addition to these reports, apoptosis in the peritoneal macrophages of parasite-infected mice was reported recently [Nishikawa et al., 2007]. This phenomenon was also confirmed in *in vitro* study, showing that *T. gondii* infection in J774A.1 mouse macrophage cells with interferon-gamma (IFN-γ) treatment activated inducible nitric oxide synthase, and consequently produced nitric oxide (NO) facilitated apoptosis [Nishikawa et al., 2007]. Such apoptosis observed in immune cells have been thought to result in the state of immunosuppression, contributing parasite survival in the host.

5. Aims of the present study

The apoptosis in the macrophages were not only due to NO, but also to the soluble fraction of parasite-infected culture, suggesting the presence of parasite-derived molecule(s) responsible for the apoptosis [Nishikawa et al., 2007]. Identification and characterization of such molecules may allow us to better understand the parasite strategy for evasion of host defense system. In this thesis, I described the general and specific features of novel identified pro-apoptotic molecule from *T. gondii*, Programmed Cell Death 5 (TgPDCD5) and discussed its significance in the

host-parasite relationship. The aim in the chapter 1 is to investigate the general characteristics of TgPDCD5 comparing with the homologues found in *Plasmodium* and in humans. The chapter 2 is to clarify cell-penetrating and apoptosis-enhancing activities of TgPDCD5 by using recombinant protein expressed in *Escherichia coli*. The aim of the chapter 3 is to evaluate the pro-apoptotic activity of endogenous TgPDCD5 by using the recombinant parasite overexpressing TgPDCD5.

Chapter 1

Cloning and general characterization of *Toxoplasma gondii* Programmed Cell Death 5 (TgPDCD5)

1-1. Introduction

Full-Toxo is a database for a full-length cDNAs from *Toxoplasma gondii* [Khan et al., 2006; http://fullmal.hgc.jp/index_tg_ajax.html]. The cDNA library was produced from the tachyzoite stage parasites using the oligo-capping method and 5'-end-one-pass sequences of random clones were determined. These expressed sequence tag (EST) sequences obtained from the database were mapped onto the genome sequences along with annotated gene structures. The cDNA clones were screened by BLAST search, and a gene showed similarity with the human apoptosis-related molecule, *Programmed Cell Death 5* (*PDCD5*) was chosen and designated as *TgPDCD5*.

The human *PDCD5* gene was cloned because expression increased during the apoptosis process induced by cytokine withdrawal in the human erythroleukemia cell line, TF-1 [Liu et al., 1999]. PDCD5 translocates from the cytoplasm to the nuclei in various apoptotic cells [Chen et al., 2001]. Overexpression of PDCD5 facilitates programmed cell death triggered by growth factor withdrawal or serum deprivation in TF-1, human stomach tumor MGC-803, and human cervical carcinoma HeLa cells [Liu et al., 1999]. It also enhances paraptotic cell death induced by TAJ/TROY, a novel member of the tumor necrosis factor receptor family [Wang et al., 2004]. Furthermore, the administration of the anti-PDCD5 antibody can suppress etoposide-induced apoptotic effects in HeLa cells [Rui et al., 2002]. These observations strongly suggest

that PDCD5 possesses a pro-apoptotic activity in mammalian cells. Recent study using recombinant PDCD5 revealed its endocytosis by human embryonic kidney HEK293, human histiocytic lymphoma U-937, and human colorectal adenocarcinoma HT-29 cells and involvement of heparan sulfate proteoglycan (HSPG)-binding motif [Wang et al., 2006].

In the BLAST search of TgPDCD5, several homologues were found also from the apicomplexan parasites *Plasmodium falciparum*, *P. yoelii*, and *P. vivax*. Among these, there was a report about *P. falciparum* apoptosis-related protein (PfARP), indicating that PfARP was strongly expressed in the ring stage and trophozoite stage, and slightly expressed in the schizont stage [Guha et al., 2007]. However, the function of this molecule is yet unknown.

In this chapter, cloning of the novel identified *TgPDCD5* gene and general characterization of its product was described.

1-2. Materials and methods

Cell lines. The mammalian cell lines used in this study were Chinese hamster ovary epithelial CHO-K1 (American Type Culture Collection [ATCC] CCL-61), mouse macrophage J774A.1 (ATCC TIB-67), and human promyeloblastic HL-60 (ATCC CCL-240). Cells were maintained as described in the ATCC instructions.

Parasites. *T. gondii* isolates RH strain and PLK strain were used in all experiments. The parasite stock was maintained *in vitro* by serial passages in a monolayer of CHO-K1 cells.

Construction and expression of recombinant TgPDCD5 (rTgPDCD5).

The full-length cDNA clone XTG05779 from Full-Toxo [Khan et al., 2006] was used as a template DNA to amplify the coding region of one complete and two truncated versions of rTgPDCD5, as shown in Fig. 1B. The DNA and amino acid sequences of TgPDCD5 are stored in the GenBank database under accession numbers AK223701 and CAJ20500. The DNA sequence of *TgPDCD5* is also accessible in the ToxoDB under number 25.m00185. DNA fragment for *TgPDCD5* was PCR amplified using the specific primers (5'-AT**C CCG GG**A TGC AGC CTG AAG AAT TCG CC-3' and 5'-AT**C TCG AG**T CAG AAG TCA TCG TCG TCG TC-3'). The PCR products were digested with *Sma*I and *Xho*I and then ligated into the glutathione *S*-transferase (GST)-fused *E. coli* expression vector pGEX-4T2 (GE Healthcare, Buckinghamshire, UK), which was digested with the same set of restriction enzymes. The nucleotide sequence was analyzed with a model ABI 3100 DNA sequencer (Applied Biosystems, Inc., Foster City, USA). The rTgPDCD5s were expressed as a fusion protein of the GST in the *E. coli* BL21 strain (GE Healthcare) according to the manufacturer's instructions. Protein concentration was measured using a BCA protein assay kit (Thermo Fisher Scientific, Inc., Rockford, USA).

All experiments with recombinant DNA and recombinant microorganisms were performed under the Guiding Principles for the Safety Assessment of Research Involving Recombinant DNA Molecules and Genetically Modified Organisms promulgated by the Obihiro University of Agriculture and Veterinary Medicine.

Production of anti-GST-TgPDCD5 serum and purification of IgG. Fifty microgram of the recombinant GST-TgPDCD5 in Freund's complete adjuvant (Sigma, St. Louis, USA) was intraperitoneally injected into 6-week-old female ddY mice (Japan SLC, Inc., Sizuoka, Japan). The same antigen in Freund's incomplete adjuvant

(Sigma) was intraperitoneally injected into the mice on days 14, 28, and 42. Immunization of a female Japanese white rabbit (CLEA Japan, Inc., Tokyo, Japan) was also performed using a similar method. Briefly, 300 μg of the antigen was inoculated intradermally for the first immunization, boosted three times by injection of 100 μg of the antigen on days 14, 28, and 42. Sera from immunized mice and the rabbit were collected 7 days after the last immunization. Immunoglobulin-G (IgG) was purified from 2 ml of rabbit sera through Protein A Chromatography Columns according to the manufacturer's instructions (Bio-Rad Laboratories, Hercules, USA). The fractions containing IgG were pooled and run on sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) to test the purity and quantity.

Animals used in this study were cared for and used under the Guiding Principles for the Care and Use of Research Animals promulgated by the Obihiro University of Agriculture and Veterinary Medicine.

Indirect fluorescent antibody test (IFAT). Localization of TgPDCD5 in the parasite was explored using coverslips of confluent CHO-K1 cells infected with *T. gondii*. The coverslips were collected at 24 h post-parasite inoculation, washed twice with phosphate-buffered saline (PBS) containing 1 mM $CaCl_2$ and $MgCl_2$ (PBS++), and then fixed with 3% paraformaldehyde (PFA) in PBS++. After washing twice with PBS++, the cells were permeabilized with 0.3% Triton X-100 in PBS++ for 5 min at room temperature. After washing, the coverslips were incubated with 3% bovine serum albumin (BSA) in PBS++ at room temperature for 30 min. The coverslips were incubated with anti-rTgPDCD5 rabbit IgG diluted at 1:100 in 3% BSA in PBS++ for 1 h at room temperature. After washing three times with PBS++, the coverslips were incubated with Alexa flour 488-conjugated goat anti-rabbit IgG (Sigma) diluted at

1:600 in 3% BSA in PBS++ for 1 h at room temperature and then washed again. After staining with propidium iodide (PI) to visualize the nuclei, the coverslips were placed on a glass slide coated with Mowiol (Sigma). The slides were examined using confocal laser scanning microscopy (TCS-NT, Leica Microsystems GmbH, Wetzlar, Germany).

Detection of native TgPDCD5 in *T. gondii* lysate. *T. gondii* lysate was prepared according to the following method. Briefly, CHO-K1 cells infected with the RH strain *T. gondii* tachyzoite were scraped from the flask and passed through a 27G needle 3 times. After filtrating through a 5.0 μm pore size nitrocellulose membrane (Millipore, Bedford, USA), the parasite number was determined. Next, 1×10^8 RH strain tachyzoites were lysed with a lysis buffer (10 mM Tris HCl [pH 7.4], 1.5 mM $MgCl_2$, 10 mM KCl, 0.5% SDS, and a complete protease inhibitor cocktail [Roche Diagnostics GmbH, Mannheim, Germany]) for 1 h at 4°C and sonicated. For the preparation of the host cell lysate, 1×10^6 HL-60, CHO-K1, and J774A.1 cells were lysed and sonicated according to the same method. Protein samples were electrophoresed in each lane of a 15% polyacrylamide gel and transferred onto a nitrocellulose membrane (Whatman GmbH, Dassel, Germany). The membrane was washed twice with a washing solution (0.05% Tween 20 in PBS) and then incubated with 5% skim milk in PBS for 30 min at room temperature. After washing twice, the membrane was incubated with anti-rTgPDCD5 mouse serum diluted at 1:100 in 0.5% skim milk in PBS for 1 h at room temperature. After washing three times, the membrane was incubated with horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG antibodies (Bethyl, Inc., Montgomery, USA) diluted at 1:8,000 in 0.5% skim milk in PBS for 1 h at room temperature. After washing three times, the protein was visualized on X-ray film using the ECL Detection Reagents (GE

Healthcare) according to the manufacturer's recommendations.

Detection of secreted TgPDCD5 from *T. gondii* excretory/secretory protein (ESP). The parasite maintained *in vitro* by serial passages in monolayers of CHO-K1 cell was harvested and washed two times with PBS. After centrifugation (500 × *g*, 10 min, 20°C), PBS was replaced by buffer A (116 mM NaCl, 5.4 mM KCl, 0.8 mM $MgSO_4$, 5.5 mM glucose, and 50 mM HEPES [pH 7.4]), and parasites were passed through a 5.0 μm pore size filter (Millipore). Finally, they were layered on a Percoll density gradient and centrifuged (500 × *g*, 20 min, 20°C). The Percoll density gradient was prepared by centrifugation (10,000 × *g*, 1 h, 20°C) of Percoll-containing buffer A, the refraction of which was adjusted to 11.4% Brix with a refractometer (Asone, Osaka, Japan). Parasites were suspended with buffer A and adjusted to 1×10^8 cells/ml. After incubation at 37°C for 0, 2, 5, 10 and 15 min, the supernatant was collected by sequential centrifugations (500 × *g*, 10 min, 20°C and then 8,400 × *g*, 10 min, 20°C) and concentrated with an NANOSEP 3K OMEGA filter (Pall Corporation, East Hills, USA). The protein concentration was calculated by spot density, which was standardized by the BSA standard on a poly vinylidene fluoride membrane (Millipore) stained with amido black. Samples were subjected to SDS-PAGE and Western blotting. The dilutions of the primary antibodies against rTgPDCD5, TgMIC2 and Tgβ-actin were 1:100, 1:500 and 1:500, respectively. The secondary antibody was diluted to 1:8,000. Secretion was quantified from Western blots using Shimadzu Phoretix (Shimadzu Corporation, Kyoto, Japan).

Immuno-electron microscopic (IEM) analyses. CHO-K1 cells infected with the RH strain *T. gondii* tachyzoite or J774A.1 treated with 4 μM of rTgPDCD5 were scraped from the flask and washed three times with PBS. Cells were fixed in 4%

PFA including 0.1% glutaraldehyde and 8% sucrose in 0.1 M phosphate buffer (pH 7.4) overnight at 4°C, washed thoroughly in 0.1 M phosphate buffer (pH 7.4) and embedded in 2% agarose. After dehydration with an ethanol series, the samples were embedded in LR Gold resin (Polysciences, Inc., Warrington, USA). Thin sections (about 80 nm thick) were cut on a Leica UCT ultramicrotome using a diamond knife and placed on nickel grids. Sections were exposed at room temperature for 30 min to 5% skim milk in PBS as a blocking agent, then incubated with anti-rTgPDCD5 rabbit IgG overnight at 4°C and subsequently incubated with 10 nm gold-labelled goat anti-rabbit IgG antibody (GE Healthcare) at room temperature for 2 h. Normal rabbit IgG was used instead of the primary antibody as a negative control. Finally, these sections were counter-stained with uranyl acetate before examination with a JEM-1011 transmission electron microscope (JEOL, Tokyo, Japan).

1-3. Results

Identification and general characterization of TgPDCD5. The cDNA clone, which is similar to the human *PDCD5* gene, an apoptosis-related molecule, was formed with 369 bp encoding a polypeptide with 122 amino acids (aa), which was predicted to be 13,707.5 Da in molecular weight and 6.87 in isoelectrical point. Using a BLASTP search, it was found that TgPDCD5 is homologous to the molecule of *Homo sapiens* (35%) and to *P. falciparum* (38%), which belongs to the same apicomplexan protozoa (Fig. 1). There was no signal peptide sequence. There was a heparan sulfate proteoglycan (HSPG)-binding motif near the C-terminus (aa 107-112), which was constructed with several basic amino acids flanked by hydrophobic residues (BXXXBB [B indicates basic residues]) [Ruoslahti, 1989; Wang et al., 2006]. This

domain was also found in human PDCD5 but not in the PfARP [Guha et al., 2007]. In a conserved domain search, a double-strand (ds) DNA-binding motif was found in the middle of TgPDCD5, which was also observed in human PDCD5 and PfARP (NCBI CDD accession number, pfam01984).

Detection of native TgPDCD5 by Western blot analysis. Mice were immunized with GST-fused rTgPDCD5 to obtain the polyclonal antibody, which recognized native TgPDCD5 in the lysate of the *T. gondii* RH strain tachyzoite at a molecular weight of 14.5 kDa with no cross-reaction with any host cell lysates tested (Fig. 2A). The lysate from *T. gondii* PLK strain, a relatively lower pathogenic Type II strain, also showed the same reactivity against the antibody in the same molecular size (data not shown). To confirm the secretion of TgPDCD5 by the parasite, the ESP from the purified extracellular parasite was tested. In Western blotting, the relative intensity of the band of TgMIC2, a typical secreted molecule from *T. gondii*, and that of TgPDCD5 were increased according to the incubation time, while Tgβ-actin, monitored as a marker for the accidental lysis of the parasite, was stable (Fig. 2B). This result indicates that TgPDCD5 was secreted from the parasite.

Localization of endogenous TgPDCD5 in *T. gondii*. To confirm the localization of native TgPDCD5 in *T. gondii*, IFAT using purified rabbit IgG against rTgPDCD5 was performed. As shown in Fig. 3A and B, the entire parasite body was faintly recognized, indicating that the protein was located in the cytoplasm and the nucleus of the intracellular tachyzoites. Even after proliferation of the parasites, the localization of the protein did not change. The extracellular parasites were also tested, resulting in the same localization (data not shown).

To determine the detailed localization of TgPDCD5, IEM analysis was

performed. Although signals recognized by the antibodies were distributed throughout the parasite body, the strongest signals were detected in the apical end of the parasites, especially near the rhoptries (Fig. 3C). Other strong signals were located within vesicle-like structures just under the parasite membrane.

1-4. Discussion

Human and *T. gondii* PDCD5 have almost the same polypeptide length (125 and 122 aa), while PfARP has much longer sequence (164 aa) due to the additional N-terminus region (Fig. 1A). Furthermore, TgPDCD5 possesses the HSPG-binding motif, which was reported to be essential for the cellular uptake of human rPDCD5 [Wang et al., 2006], but which is lacking in the homologue in *P. falciparum*, PfARP [Guha et al., 2007]. Thus, the molecular features of TgPDCD5 might be more similar to human PDCD5 than to PfARP, even though *T. gondii* and *P. falciparum* are phylogenetically very similar. The cytosolic localization of TgPDCD5 was consistent with those of human PDCD5 and PfARP in the normal condition [Chen et al., 2001; Guha et al., 2007]. However, the existence of dsDNA-binding motif suggested the possibility that this protein functions in the nucleus or other organelles with DNA such as mitochondrion and apicoplast. Together with that translocation of human PDCD5 to the nucleus during apoptosis had been reported [Chen et al., 2001], TgPDCD5 might not be a strict cytosolic protein.

Many kinds of secreted molecules from apicomplexan parasites have been identified, including microneme, dense-granule, and rhoptry proteins [Cesbron-Delauw, 1994; Carrhthers, 1999; Soldati et al., 2001]. Most models for the secretion of these proteins were based on the classic pathway involving the endoplasmic reticulum and

Golgi. According to the previously identified molecules, appropriate signal sequences in the N-terminus were required for the processing and transporting of the secreted proteins via the classic pathway [Joiner and Roos, 2002]. In addition to the result of secretion assay (Fig. 2B), the localization of TgPDCD5 near the rhoptry or in the vesicle-like structure just under the cellular membrane also confirmed its secretion (Fig. 3C). However, no domain was predicted to be the signal sequence in TgPDCD5 (Fig. 1A), suggesting that this molecule was secreted not via the classic pathway but via an unknown mechanism. A study using calcium ionophore A23187 indicated that the secretion of TgPDCD5 was not dependent on the elevation of intracellular calcium ions (data not shown) suggesting that TgPDCD5 secretion occurred in a manner different from that of microneme proteins such as TgMIC2 [Carruthers and Sibley, 1999; Vieira and Moreno, 2000]. To determine the secretion pathway of TgPDCD5, further study is required.

1-5. Summary

The *TgPDCD5* gene, which is a homologue of human apoptosis-related molecule PDCD5 was cloned from cDNA database and characterized. The predicted amino acid sequence of TgPDCD5 consisted with 122 aa in length, having 35% and 38% similarity to the homologues in *H. sapiens* and *P. falciparum*. This protein has dsDNA-binding motif in the middle part and HSPG-binding motif in the C-terminus region. The native TgPDCD5 was recognized by the polyclonal antibodies in the parasite lysate as a 14.5 kDa protein. This protein localized in cytosol in intracellular and extracellular tachyzoites. According to the IEM analysis, the strongest signals localized in the vesicle-like structures near the rhoptries or just under the parasite membrane. The secretion of this protein was also confirmed by Western blotting of ESP.

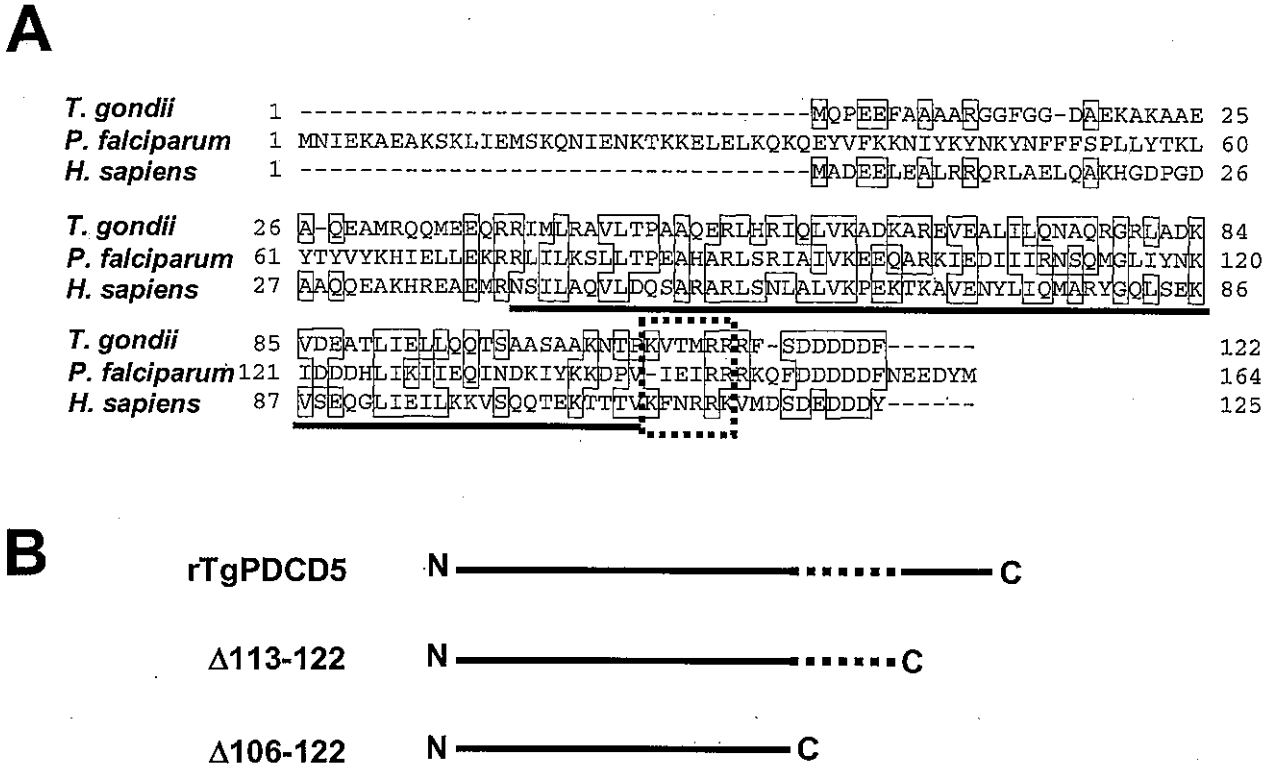

Fig. 1. Amino acid sequences of TgPDCD5 and its homologues.
(A) Alignment of PDCD5 homologues of *T. gondii*, *P. falciparum*, and *H. sapiens* (GenBank accession numbers XP_001351965 and CAG33215, respectively). Underline, double-strand DNA-binding domain; dashed-line box, HSPG-binding motif (BXXXBB).
(B) Structure of a series of rTgPDCD5. Dashed line, HSPG-binding motif.

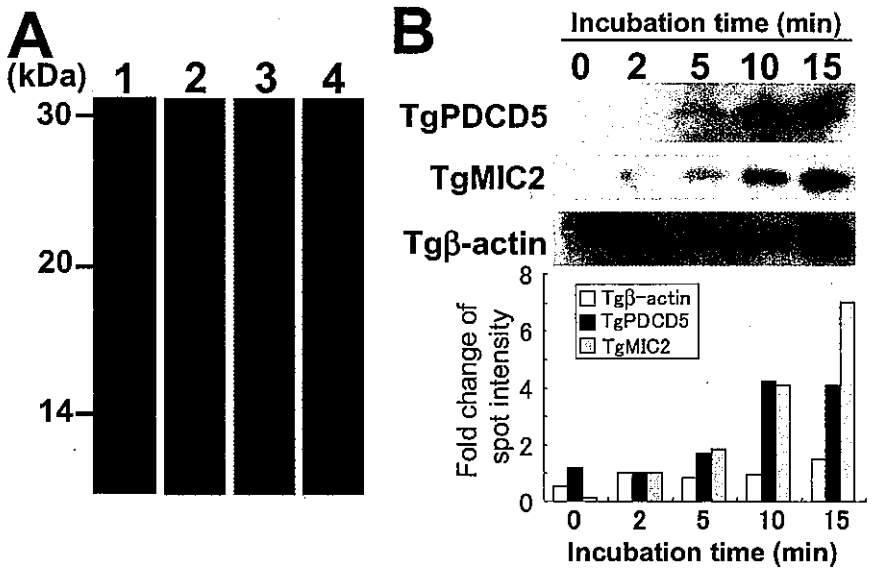

Fig. 2. Western blot analyses for the detection of native TgPDCD5.
(A) Lysates of parasite and host cells were electrophoresed in 15% polyacrylamide gel and transferred onto a nitrocellulose membrane. They were then probed with the mouse anti-rTgPDCD5 antibody, followed by the HRP-conjugated goat anti-mouse IgG antibody. Lane 1, *T. gondii* lysate; lane 2, CHO-K1 cell lysate; lane 3, J774A.1 cell lysate; lane 4, HL-60 cell lysate.
(B) TgPDCD5 detected in a secreted protein population. A medium cultured with purified *T. gondii* tachyzoite for 0, 2, 5, 10 and 15 min was electrophoresed, transferred, and probed as described above. The blot was also immuno-stained with anti-TgMIC2 antibody as a positive control and with anti-Tgβ-actin antibody as a marker of accidental lysis. The histogram indicates the fold change of spot density.

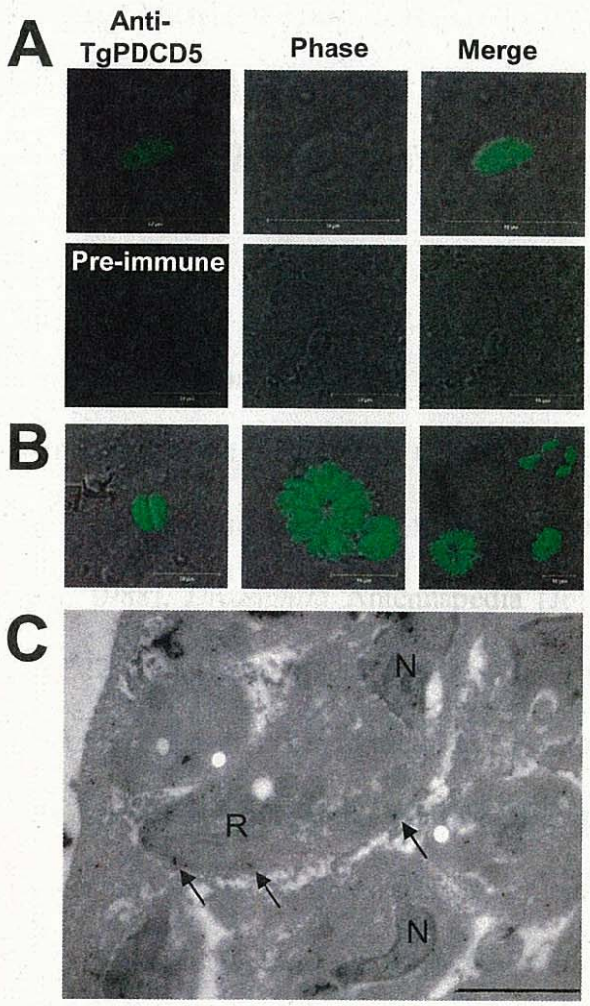

Fig. 3. Localization of native TgPDCD5 in *T. gondii*-infected CHO-K1 cells.
(A) IFAT images of TgPDCD5 localization. *T. gondii*-infected CHO-K1 cells on the coverslips were fixed and immuno-stained with rabbit anti-rTgPDCD5 IgG and Alexa flour 488-conjugated goat anti-rabbit IgG. Bar, 10 μm.
(B) Merged images from proliferating parasites.
(C) IEM images of TgPDCD5 localization. *T. gondii*-infected CHO-K1 cells in the ultrathin sections were immuno-stained with rabbit anti-rTgPDCD5 IgG and gold-labelled goat anti-rabbit IgG antibody. Arrows indicate signals detected in the vesicle-like structures. R, rhoptries; N, nucleus. Bar, 1 μm.

Chapter 2
HSPG-binding motif-dependent internalization of recombinant TgPDCD5 by host cells and apoptosis enhancement

2-1. Introduction

The endocytosis after addition of rPDCD5 was reportedly lipid raft-dependent, and clathrin-independent [Wang et al., 2006]. This machinery of protein transport was previously found in the human immunodeficiency virus Tat [Frankel and Pabo, 1988; Green and Loewenstein, 1988], *Drosophila* Antennapedia [Joliot et al., 1991], and herpes simplex virus VP22 [Elliot and O'Hare, 1997]. These translocatory proteins possess strong cell-penetrating capacity, hence they have been used in drug delivery and gene therapy studies. They shared several common features: they are released from cells by a pathway distinct from the recognized secretory routes involving a secretion signals [Helland et al., 1991; Joliot et al., 1998]; they bind to target cells in a receptor-independent manner; and each of them has a highly basic region that appears to mediate the ability of these proteins to bind to polyanions, such as heparin/heparan sulfate, polysialic acid, and nucleic acids. Having these features in common, TgPDCD5 may be one of the translocatory proteins like human PDCD5 and other proteins mentioned above.

Exogenously added human rPDCD5 to the culture medium can also enhance apoptosis triggered by growth factor deprivation in TF-1 cells or serum deprivation in HL-60 cells [Zhang et al., 2006]. Combined with the cell penetrating capacity [Wang et al., 2006], they provided a novel mechanism for the positive regulation of apoptosis:

after upregulation of endogenous PDCD5 expression, this protein can be released by dead cells and then reuptaken by other cells via lipid raft-endocytosis to promote further apoptosis. In this chapter, recombinant TgPDCD5 protein was used to evaluate whether it is uptaken by the host cells and enhance host cell apoptosis.

2-2. Materials and methods

Cell lines. The mammalian cell lines used in this study were Chinese hamster ovary epithelial CHO-K1 (American Type Culture Collection [ATCC] CCL-61), mouse macrophage J774A.1 (ATCC TIB-67), and human promyeloblastic HL-60 (ATCC CCL-240). Cells were maintained as described in the ATCC instructions.

Parasites. A *T. gondii* isolate RH strain which expresses green fluorescent protein (GFP) [Nishikawa et al., 2003] was used in all experiments. The parasite stock was maintained *in vitro* by serial passages in a monolayer of CHO-K1 cells.

Preparation of full-length and truncated version of rTgPDCD5. The PCR amplification for the full-length TgPDCD5 gene was performed as described in the chapter 1. The following primer sets were used to amplify the DNA fragments for the two truncated TgPDCD5s; Δ113-122 and Δ106-122. For Δ113-122, which was truncated at 9 aa of the C terminus of rTgPDCD5, the primers (5'-AT**C CCG GG**A TGC AGC CTG AAG AAT TCG CC-3' and 5'-AT**C TCG AG**T TAC CGT CTC ATA GTG ACT TTC GG-3') were used. For Δ106-122, which was truncated at 16 aa of the C terminus, the primers (5'-AT**C CCG GG**A TGC AGC CTG AAG AAT TCG CC-3' and 5'-AT**C TCG AG**T TAC GTA TTC TTC GCC GCA GAC GC-3') were used. The PCR products were digested with *Sma*I and *Xho*I and then ligated into the GST-fused *E.*

coli expression vector pGEX-4T2 (GE Healthcare), which was digested with the same set of restriction enzymes. The nucleotide sequences were analyzed with a model ABI 3100 DNA sequencer (Applied Biosystems). One full-length and two truncated rTgPDCD5s were expressed as a fusion protein of the GST in the *E. coli* BL21 strain (GE Healthcare) according to the manufacturer's instructions. GST tags of the recombinant proteins were removed with thrombin protease (GE Healthcare) according to the manufacturer's recommendation. Proteins were purified with Detoxi-GelTM Endotoxin Removing Gel (Thermo Fisher Scientific, Inc.), and the concentration was measured using a BCA protein assay kit (Thermo Fisher Scientific, Inc.).

All experiments with recombinant DNA and recombinant microorganisms were performed under the Guiding Principles for the Safety Assessment of Research Involving Recombinant DNA Molecules and Genetically Modified Organisms promulgated by the Obihiro University of Agriculture and Veterinary Medicine.

Observation of internalized rTgPDCD5. J774A.1 cells (1×10^5) were seeded on coverslips and incubated for 8 h until they adhered. After 12 h incubation of the cells with 4 μM of rTgPDCD5, Δ113-122 and Δ106-122, or GST, the coverslips were washed 3 times with PBS++, fixed with 3% PFA in PBS++, and used for immunostaining with anti-rTgPDCD5 mouse serum and Alexa flour 488-conjugated goat anti-mouse IgG (Sigma). After staining with PI to visualize the nuclei, the coverslips were placed on a glass slide coated with Mowiol (Sigma). The slides were examined using confocal laser scanning microscopy (TCS-NT, Leica Microsystems GmbH).

Apoptosis induction. J774A.1 (2.5×10^4) cells were seeded in a 48-well plate and incubated for 8 h until they adhered to the bottom. Cells were incubated with

differing concentrations of rTgPDCD5, Δ113-122, Δ106-122, or GST for 12 h. Forty μg/ml of etoposide (Sigma) was added to the culture for apoptosis induction. After 6 h of incubation at 37°C, the cells were collected. For HL-60 cells, 5×10^5 cells were seeded and incubated with the same concentration of recombinant proteins and etoposide for 2 h. Method for apoptosis induction in cooperation with IFN-γ was described previously [Nishikawa et al., 2007]. Briefly, 2.5×10^5 J774A.1 cells were inoculated with 5×10^6 *T. gondii* RH strain expressing GFP, then incubated with or without 10 unit/ml of IFN-γ (Thermo Fisher Scientific, Inc.) and 2 μM of rTgPDCD5, Δ113-122, Δ106-122, or GST for 24 h at 37°C.

Detection of apoptotic cells. For the detection of apoptosis, cells were washed two times with PBS, re-suspended in PBS, and placed on glass slides. The slides were dried and then fixed with 4% PFA in PBS before use. After washing twice with PBS, the slides were permeabilized with 0.1% Triton X-100 in 0.1% sodium citrate for 2 min on ice. After washing, the slides were incubated with an *In Situ* Cell Death Detection Kit, TMR red (Roche Diagnostics) at 37°C for 1 h to stain apoptotic cells with the terminal deoxynucleotidyltransferase-mediated dUTP-biotin nick-end labeling (TUNEL) method. After washing twice, the slides were examined using a fluorescence microscope (Nikon, Tokyo, Japan). The mean ratio of apoptotic cells was calculated by counting TUNEL-positive cells and total cells in three individual fields from each slide. Each field contained at least three hundred cells. The statistical significance was calculated with the Student's *t*-test from the mean and S.D. of triplicate samples.

Caspase-3 measurement. For the caspase-3 assay, cells treated with rTgPDCD5 or GST were lysed and the protein concentration was quantified.

Caspase-3 activity was measured using Caspase-3/CPP32 Colorimetric Assay Kit (Medical and Biological Laboratories, Nagoya, Japan) according to the manufacturer's instructions. Absorbance at 405 nm of each condition was measured and standardized with that of cells not treated with rTgPDCD5 or etoposide. The ratio was expressed as relative caspase-3 activity. The statistical significance was calculated with the Student's *t*-test from the mean and S.D. of triplicate samples.

2-3. Results

Internalization of rTgPDCD5 by host cells. rTgPDCD5 or GST was added to the culture medium of J774A.1 mouse macrophage cells. An IFAT showed that rTgPDCD5 was located in the cytosol after twelve hours incubation, whereas GST was not detected, indicating the specific internalization of rTgPDCD5 by the host cells (Fig. 4A). The result of IEM analysis showed that internalized rTgPDCD5 was localized in the small vesicles in the cytosol (Fig. 4B).

In order to confirm the involvement of HSPG-binding motif in the rTgPDCD5 penetration, two truncated rTgPDCD5s, Δ113-122 which has the HSPG-binding motif, and Δ106-122 which does not have the motif, were constructed. To confirm the reactivity of the anti-rTgPDCD5 antibody against these recombinant proteins, Western blot analysis was performed (Fig. 4C). The full-length rTgPDCD5, Δ113-122 and Δ106-122 were equally recognized by the anti-rTgPDCD5 antibody, while BSA, a negative control, was not recognized. These truncated proteins were tested whether they can be internalized. As shown in Fig. 4D, Δ113-122 was still internalized by the cells, while Δ106-122 was not, indicating that seven amino acids located from position 106 to position 112 corresponding to the HSPG-binding motif were essential for the

internalization of rTgPDCD5.

Apoptosis enhancement by rTgPDCD5 in J774A.1 and HL-60 cells. The rTgPDCD5 was evaluated whether it shows pro-apoptotic activity as the human homologue. In addition to the J774A.1 cell, the human promyeloblastic cell, HL-60, was tested because this cell line was used in a previous study in which human PDCD5 enhanced apoptosis [Zhang et al., 2000]. J774A.1 cells were pre-cultured with rTgPDCD5, and apoptosis was triggered by etoposide, the topoisomerase II inhibitor [Kaufmann, 1989]. Genomic DNA fragmentation in the treated cells was detected with TUNEL staining. The J774A.1 cells treated with 40 μg/ml etoposide were 5.95 ± 0.39% TUNEL-positive, while only 0.06 ± 0.11% of the cells were positive in the absence of etoposide. The rates of apoptosis in cells increased with the addition of rTgPDCD5 to the culture in a dose-dependent manner up to 12.28 ± 1.13% ($P < 0.05$) (Fig. 5A). However, the cells treated with rTgPDCD5 did not increase apoptosis in the absence of etoposide, suggesting that rTgPDCD5 alone could not initiate apoptosis. Caspase-3 activity, a major player in the apoptotic process, was also tested for the confirmation of apoptosis. As shown in Fig. 5C, caspase-3 activity of rTgPDCD5-treated J774A.1 gradually increased in proportion to the increased TUNEL-positive cells. This result indicates that rTgPDCD5 has a pro-apoptotic effect on the cells.

Apoptosis enhancement was also observed when J774A.1 cells were treated with Δ113-122 (8.09 ± 0.18%, $P < 0.01$) at almost the same level as full-length rTgPDCD5 (8.70 ± 0.49%, $P < 0.01$). However, Δ106-122 had no enhancing effect (4.87 ± 0.19%), like the negative controls, PBS (4.74 ± 0.56%) and GST (4.55 ± 0.81%) (Fig. 6A), indicating that an HSPG-binding motif, which was essential for the

internalization of rTgPDCD5, was also required for enhanced apoptosis activity. Apoptosis enhancement by rTgPDCD5 and the requirement of the HSPG-binding motif was confirmed also in the HL-60 cells ($P < 0.05$) (Figs. 5B, D, and 6B).

Apoptosis induction by rTgPDCD5 in cooperation with IFN-γ in J774A.1 cells. The previous study by Nishikawa et al. indicated that *T. gondii* infection resulted in the induction of apoptosis in uninfected bystander cells both *in vitro* and *in vivo* in the presence of IFN-γ and consequently produced NO [2007]. Since the pro-apoptotic activity of rTgPDCD5 was confirmed in the apoptosis assay using etoposide, I next evaluated whether this molecule was active in causing apoptosis in cooperation with IFN-γ, as observed in *T. gondii* infection (Fig. 7). In the presence of IFN-γ, the parasite-infected group showed 20.27 ± 4.76% of apoptotic cells, while the mock-infected group showed only 2.29 ± 0.79% ($P < 0.01$). Cells treated with rTgPDCD5 and IFN-γ showed significantly higher rates of apoptosis (17.40 ± 2.16%, $P < 0.01$) compared to the untreated group. Involvement of the HSPG-binding motif in apoptosis induction was also confirmed using Δ113-122 (11.90 ± 3.24%) and Δ106-122 (3.49 ± 1.34%). These results indicated that TgPDCD5 caused apoptosis in J774A.1 cells in cooperation with IFN-γ in an HSPG-binding motif-dependent manner.

The recombinant proteins and IFN-γ were applied to *T. gondii*-infected cells. Apoptosis caused by the parasite and IFN-γ was up-regulated by the addition of rTgPDCD5 (17.49 ± 0.90%) and Δ113-122 (17.93 ± 1.41%) but not by Δ106-122 (10.98 ± 0.54%) and GST (9.45 ± 0.20%) (Table 1). The rate of positive cells in both GFP and TUNEL staining did not change significantly (0.65 ± 0.33% to 1.20 ± 0.18%) even after the addition of the recombinant protein, indicating that apoptosis occurred almost exclusively in uninfected cells.

2-4. Discussion

The penetration of rTgPDCD5 into the host cells observed in IFAT and IEM analyses (Fig. 4A and B) suggested a direct interaction of the protein with host cells. The ways by which the secreted molecules from parasite interact with host cells can be classified into three groups. The first way is the binding of parasite-secreted proteins to the host cell surface, including the major secreted protein MIC2, which binds to the intracellular adhesion molecule 1 of the host cells [Nagineni et al., 2000; Barragan et al., 2005; Brossier and Sibley, 2005]. This interaction makes it possible for the parasites to migrate across polarized epithelial cells. The second way is observed in some rhoptry proteins or dense-granule proteins that associate with the parasitophorous vacuole (PV) membrane. They expose their partial amino acids towards host cell cytoplasm [Beckers et al., 1994; Sinai and Joiner, 2001; Carey et al., 2004; El Hajj et al., 2007]. The third way is seen in the recent report on ROP16, indicating that the secreted protein reaches the host-cell nucleus, activating the signal transducer and activator of transcription pathways and bringing downstream effects on a host cytokine, interleukin-12 (IL-12) [Saeji et al., 2007]. The machinery by which TgPDCD5 penetrates into the host cells is distinct from those mentioned above but may be common with other lipid raft-endocytosed proteins [Frankel 1988; Green 1988; Joliot 1991; Elliot 1997], because of the requirement of HSPG-binding motif for the uptake.

HSPG-binding motif-dependency was also the case in pro-apoptotic effect of this molecule (Figs. 6, and 7). Considering the role of this motif in protein penetration, the uptake of rTgPDCD5 seems to lead the cells to enhanced apoptosis directly or indirectly. The apoptosis induction by rTgPDCD5 with IFN-γ indicated that the

capacity of this protein was not limited to the artificial apoptosis model using etoposide, but may be involved in the macrophage apoptosis in parasite-infection reported previously [Hisaeda et al., 1997; Nishikawa et al., 2007]. The possible explanation for the apoptosis only observed in uninfected bystander cells but not in parasite-infected cells is yet unknown but can be speculated as follows: the pro-apoptotic effect of TgPDCD5 may not be enough to cause apoptosis; instead, the effect could be canceled by anti-apoptotic effects, as reported by other groups [Goebel et al., 2001; Nishikawa et al., 2002; Molestina et al., 2003; Carmen et al., 2006; Keller et al., 2006].

2-5. Summary

The capacity of TgPDCD5 to penetrate the host cells and to enhance apoptosis was evaluated. Studies using recombinant TgPDCD5 demonstrated that host cells internalize the molecule in a HSPG-binding motif-dependent manner. Furthermore, the addition of rTgPDCD5 to culture medium resulted in the enhancement of host-cell apoptosis triggered by etoposide in macrophage cell line J774A.1 and leukemic cell line HL-60 cells. Additionally, rTgPDCD5 induced apoptosis in J774A.1 cells in the presence of IFN-γ. These results suggested that TgPDCD5 might be one of the soluble factors responsible for the apoptosis observed in macrophages of parasite-infected mice. Since the apoptosis-enhancement was also HSPG-binding motif-dependently occurred, the internalized rTgPDCD5 directly or indirectly lead the cells to apoptosis. This was the first report of the parasite-derived molecule that exerts a pro-apoptotic activity on host cells.

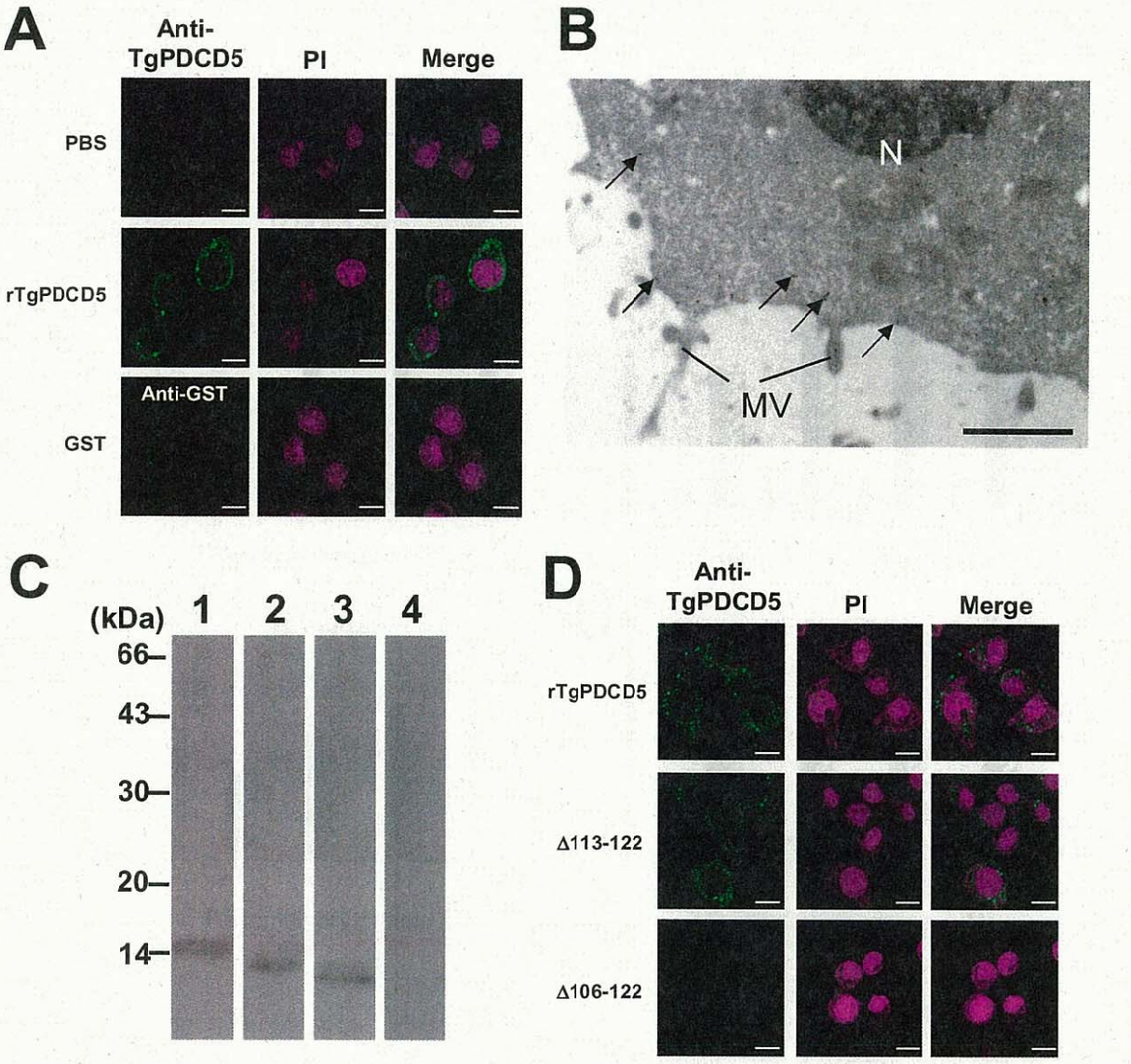

Fig. 4. J774A.1 cells specifically internalize rTgPDCD5 in an HSPG-binding motif-dependent manner.

(A) Specific internalization of rTgPDCD5 by J774A.1 cells. Cells were incubated with 4 μM of full-length rTgPDCD5 or GST, fixed, and stained with the mouse anti-rTgPDCD5 antibody or anti-GST antibody, followed by Alexa Flour 488-conjugated goat anti-mouse IgG antibody and PI. Bar, 10 μm. Internalization was confirmed by IFAT using rabbit anti-rTgPDCD5 antibody also (data not shown).

(B) IEM image of internalized rTgPDCD5. Protein-treated J774A.1 cells in the ultrathin sections were immuno-stained with rabbit anti-rTgPDCD5 IgG antibody and gold-labelled goat anti-rabbit IgG antibody. Arrows indicate signals detected in the vesicles in the cytosol. N, nucleus; MV, microvillus. Bar, 1 μm.

(C) Mouse anti-rTgPDCD5 antibody recognized full-length rTgPDCD5, Δ113-122, and Δ106-122. Each recombinant protein was electrophoresed in a 15% polyacrylamide gel and transferred onto a nitrocellulose membrane. It was then probed with the mouse anti-rTgPDCD5 antibody, followed by HRP-conjugated goat anti-mouse IgG antibody. Lane 1, full-length rTgPDCD5; lane 2, Δ113-122; lane 3, Δ106-122; lane 4, BSA.

(D) Internalization of rTgPDCD5 was dependent on the HSPG-binding motif. J774A.1 cells were incubated with 4 μM of full-length rTgPDCD5, Δ113-122, or Δ106-122. They were then fixed and immuno-stained as described above. Bar, 10 μm.

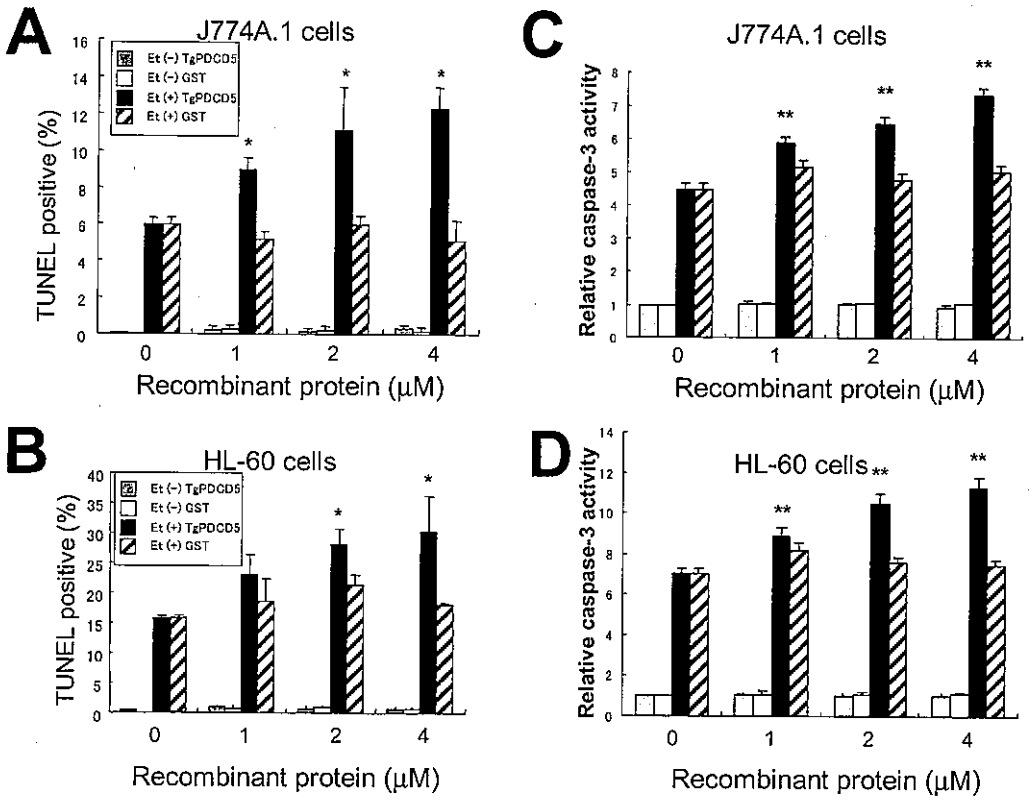

Fig. 5. rTgPDCD5 enhances host-cell apoptosis triggered by etoposide.
(A) Effects of rTgPDCD5 on apoptosis enhancement in J774A.1 cells. After incubation with 1, 2, and 4 μM of full-length rTgPDCD5 or GST for 12 h, the cells were treated with or without 40 μg/ml etoposide for 6 h.
(B) Effects of rTgPDCD5 on apoptosis enhancement in HL-60 cells. Cells were treated with 1, 2, and 4 μM of rTgPDCD5 or GST and 40 μg/ml etoposide for 2 h. After the treatment of the cells, genomic DNA fragmentation was detected with TUNEL staining, and the ratio of apoptotic cells was determined. The histograms indicate the mean and S.D. of triplicate samples.
(C) Effects of rTgPDCD5 on caspase-3 activity in J774A.1 cells.
(D) Effects of rTgPDCD5 on caspase-3 activity in HL-60 cells. Cells incubated with 1, 2, and 4 μM of full-length rTgPDCD5 or GST and etoposide were lysed and subjected to the caspase-3 assay. Absorbance at 405 nm was measured and expressed as the mean of fold changes ± S.D. of triplicates. Statistical significance was calculated with the Student's *t*-test. * $P < 0.05$, ** $P < 0.01$. Et, etoposide.

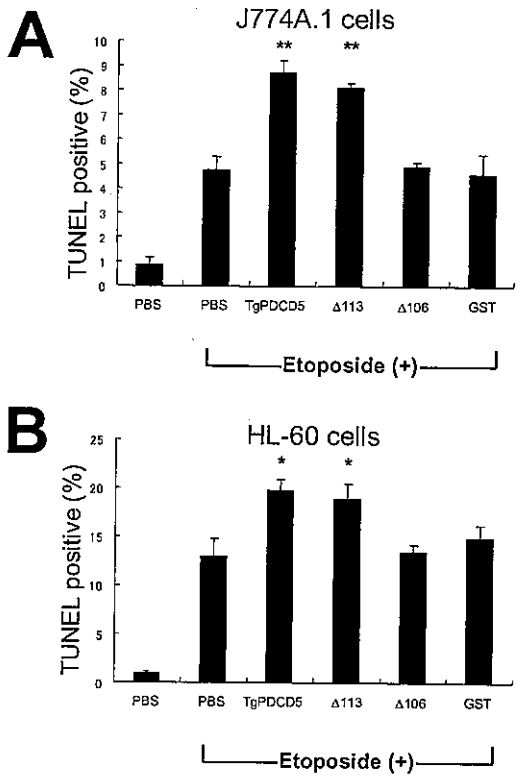

Fig. 6. Pro-apoptotic effect of rTgPDCD5 was HSPG-binding motif-dependent.
(A) Effects of the HSPG-binding motif on apoptosis enhancement in J774A.1 cells. After incubation with 2 μM of each recombinant protein for 12 h, cells were treated with 40 μg/ml etoposide for 6 h.
(B) Effects of the HSPG-binding motif on apoptosis enhancement in HL-60 cells. Cells were treated with 2 μM of each recombinant protein and 40 μg/ml etoposide for 2 h. After the treatment of the cells, genomic DNA fragmentation was detected with TUNEL staining, and the ratio of apoptotic cells was determined. The histograms indicate the mean and S.D. of triplicate samples. Statistical significance was calculated with the Student's *t*-test. * $P < 0.05$. Δ113, Δ113-122; Δ106, Δ106-122.

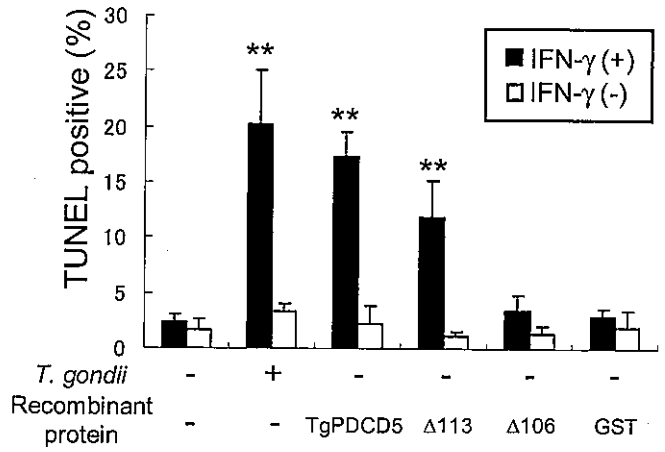

Fig. 7. Apoptosis induction by rTgPDCD5 in cooperation with IFN-γ.
J774A.1 cells were incubated with or without 10 unit/ml IFN-γ and 2 μM of each recombinant protein for 24 h. As a positive control of apoptosis induction, cells were inoculated with *T. gondii* RH strain expressing GFP at moi = 2.0. After the cells were treated, genomic DNA fragmentation was detected with TUNEL staining, and the ratio of apoptotic cells was determined. The histograms indicate the mean and S.D. of triplicate samples. Statistical significance was calculated with the Student's *t*-test. ** $P < 0.01$. Δ113, Δ113-122; Δ106, Δ106-122.

Table 1. Apoptosis induction by rTgPDCD5 and IFN-γ in *T. gondii*-infected cells.

J774A.1 cells	Recombinant protein				
	-	rTgPDCD5	Δ113-122	Δ106-122	GST
% of GFP^+	25.20 ± 1.12	23.56 ± 2.00	21.56 ± 4.36	25.39 ± 2.99	27.19 ± 1.40
% of $TUNEL^+$	12.28 ± 2.00	17.49 ± 0.90**	17.93 ± 1.41**	10.98 ± 0.54	9.45 ± 0.20
% of GFP^+ $TUNEL^+$	1.20 ± 0.18	0.93 ± 0.46	0.65 ± 0.33	0.78 ± 0.25	1.12 ± 0.20

J774A.1 cells were inoculated with *T. gondii* RH strain expressing GFP at moi = 2.0 and then incubated with 10 unit/ml IFN-γ and 2 μM of each recombinant protein for 24 h. After TUNEL staining, the ratio of apoptotic cells to parasite-infected cells was determined by counting TUNEL+ cells and GFP+ cells. Results are expressed as the mean and S.D. of triplicates. Statistical significance was calculated with the Student's *t*-test. ** $P < 0.01$.

Chapter 3

Evaluation of pro-apoptotic activity of endogenous TgPDCD5 during infection *in vitro* using overexpressing parasite

3-1. Introduction

Overexpression system of homologous or heterologous protein in *T. gondii* is a powerful method for the investigation of host-parasite interactions and functional analysis of certain molecules. Methods for the transient and stable transfection of DNA construct into the parasite have been established by many groups [Donald and Roos, 1993; Kim et al., 1993; Soldati and Boothroyd, 1993; Sibley et al., 1994; Messina et al., 1995], and there has been remarkable progress in the development of multiple selectable markers and inducible promotors [Roos et al., 1994; Soldati, 1996; Boothroyd et al., 1997; Roos et al., 1997; Ajioka, 1998; Striepen et al., 1998; Meissner et al., 2002; Striepen et al., 2002].

In the chapter 2, the capacity of rTgPDCD5 to penetrate into the host cells and to enhance apoptosis was confirmed by using recombinant protein. In order to clarify whether the endogenous TgPDCD5 is really involved in the host cell apoptosis during *T. gondii* infection, the recombinant parasite which overexpresses TgPDCD5 was established. This chapter discussed the function of endogenous TgPDCD5, by using the recombinant parasite to evaluate its activity to induce apoptosis in host macrophages.

3-2. Materials and methods

Cell lines. The mammalian cell lines used in this study were Chinese

hamster ovary epithelial CHO-K1 (American Type Culture Collection [ATCC] CCL-61), mouse macrophage J774A.1 (ATCC TIB-67), monkey kidney adherent fibroblasts Vero (ATCC CCL-81). Cells were maintained as described in the ATCC instructions.

Parasites. *T. gondii* isolates used in this study were PLK wild type strain (PLK-WT), the recombinant PLK strain which expresses green fluorescent protein (PLK-GFP) [Nishikawa et al., 2003], and the recombinant PLK strains which overexpresses TgPDCD5 (PLK-TgPDCD5) or Δ106-122 (PLK-Δ106) (see below). The parasite stock was maintained *in vitro* by serial passages in a monolayer of CHO-K1 cells.

Construction of transfer vector. The plasmid for overexpression in *T. gondii*, pDMG was constructed as follows. The 5' flanking region of the *dense granule protein 1 (GRA1)* gene and the 3' flanking region of the *GRA2* gene were PCR amplified from the plasmid GRAGFP (kindly provided by Dr. J.C.Boothroyd, Stanford University) by using primers for GRA1-5'UTR (5'-AC**G TCG ACC** GAA GGC TGT AGT ACT-3' and 5'-AC**A AGC TTC** TTG CTT GAT TTC TTC-3' and for GRA2-3'UTR (5'-AC**G GAT CC**A AGA CTA CGA CGA AAG-3' and 5'-AC**T CTA GA**G TCG ACT GGA ACT ACG-3'), respectively. The PCR products of GRA1-5'UTR and GRA2-3'UTR were digested with *Sal*I-*Hin*dIII and *Bam*HI-*Xba*I, respectively, and cloned into the corresponding sites of pBluescript KS+, and pKS/GRA5'-GRA3' was obtained. The GFP coding sequence was PCR amplified from plasmid GRAGFP by using specific primers (5'-AC**G AAT TC**C AAG ATG CAT AAA GGA-3' and 5'-AC**G AAT TC**T TAT TTG TAT AGT TCA-3'). The resulting PCR product was digested with *Eco*RI, and cloned into *Eco*RI site between GRA1-5'UTR

and GRA2-3'UTR of pKS/GRA5'-GRA3', and obtained pKS/GRA-GFP. The *Sal*I-*Xba*I fragment (GRA5'-GFP-GRA3') from pKS/GRA-GFP was blunt ended by Klenow fragment and inserted into blunt ended *Xba*I site of pDHFR-TSc3 [Donald and Roos, 1993] (kindly provided by Dr. D.S.Roos, University of Pennsylvania), and hence, pDHFR-GFP was obtained. The *Sal*I-*Xba*I fragment (GRA5'-multicloning sites [MCS]-GRA3') from pKS/GRA5'-GRA3' was blunt ended by Klenow fragment and inserted into the blunt ended *Spe*I site of pDHFR-GFP. The plasmid with the DHFR-GRA5'-MCS-GRA3'-GRA5'-GFP-GRA3' fragment was designated as pDMG.

The DNA fragment for full-length TgPDCD5 was PCR amplified from the full-length cDNA clone XTG05779 of Full-Toxo [Khan et al., 2006] with the primers (5'-AT**C CAT GG**A TGC AGC CTG AAG AAT TCG CC-3' and 5'-TA**G CTA GC**G AAG TCA TCG TCG TCG TCA CT-3'). For the Δ106-122, the reverse primer (5'-TA**G CTA GC**A TTC TTC GCC GCA GAC GCC GC-3') was used. The resulting products were digested with *Nco*I and *Nhe*I and inserted into the same sites of pHXNTPHA (kindly provided by K.A.Joiner, Yale University), and hence, pHXNTP-TgPDCD5 (or Δ106)-HA was obtained. TgPDCD5 (or Δ106) with HA-tag (YPYDVPDYA) was obtained from pHXNTP-TgPDCD5 (or Δ106)-HA by *Nco*I and *Bgl*II digestion. This fragment was blunt ended by Klenow fragment and inserted into the *Eco*RV site of pDMG; pDMG-TgPDCD5 and pDMG-Δ106 were obtained.

Transfection and selection of *T. gondii* overexpressing TgPDCD5 or Δ106-122. Electroporation of tachyzoites was performed as described [Sibley et al., 1994]. Briefly, purified *T. gondii* PLK tachyzoites were resuspended at 10^7 cells/ml with cytomix buffer (120 mM KCl, 0.15 mM $CaCl_2$, 10 mM K_2HPO_4-KH_2PO_4, 2 mM EDTA, 5 mM $MgCl_2$, 25 mM HEPES [pH 7.6]), supplemented with 2 mM ATP and 5

mM glutathione. Cells were transferred to a 2 mm-gap cuvette and electroporated with 2.0 kV at 50 Ω with a Gene Pulser II (BioRad Laboratories). After transfection, tachyzoites were allowed to infect Vero cells in a drug-free culture medium for 18 h to permit the phenotypic expression of the dihydrofolate reductase-thymidylate synthase and GFP genes as selectable markers, at which time pyrimethamine was added at a final concentration of 1 μM. Polyclonal transfected pyrimethamine-resistant tachyzoite cultures were subjected to plaque purification. The cultures were passaged three times in the medium containing 1% agarose and a single plaque was obtained. The recombinant *T. gondii* clones constructed using transfer vectors, pDMG-TgPDCD5 and pDMG-Δ106 were designated as, PLK-TgPDCD5 and PLK-Δ106, respectively.

All experiments with recombinant DNA and recombinant microorganisms were performed under the Guiding Principles for the Safety Assessment of Research Involving Recombinant DNA Molecules and Genetically Modified Organisms promulgated by the Obihiro University of Agriculture and Veterinary Medicine.

IFAT. Confluent CHO-K1 cells on the coverslips were inoculated with PLK-TgPDCD5 or PLK-Δ106. The coverslips were collected at 24 h post-parasite inoculation, washed 3 times with PBS++, fixed with 3% PFA in PBS++, and used for immunostaining with anti-HA monoclonal antibody (Covance Research Products, Inc., Berkeley, USA) and Alexa flour 594-conjugated goat anti-mouse IgG (Sigma). The coverslips were placed on a glass slide coated with Mowiol (Sigma). The slides were examined using confocal laser scanning microscopy (TCS-NT, Leica Microsystems GmbH).

Western blot analysis of PLK-TgPDCD5 and PLK-Δ106. The parasite lysate was prepared according to the following method. Briefly, 1×10^8 PLK-WT,

PLK-TgPDCD5 and PLK-Δ106 tachyzoites were harvested and lysed with a lysis buffer as described in chapter 1. Protein samples were electrophoresed in each lane of a 15% polyacrylamide gel and transferred onto a nitrocellulose membrane (Whatman GmbH). After washing twice with a washing solution, the membrane was incubated with 5% skim milk in PBS for 30 min at room temperature. After washing twice, the membrane was incubated with anti-rTgPDCD5 mouse serum or anti-HA mouse monoclonal antibody for 1 h at room temperature. The dilution of primary antibodies were 1:100 for anti-rTgPDCD5 mouse serum and 1:1,000 for anti-HA mouse monoclonal antibody, respectively. After washing three times, the membrane was incubated with HRP-conjugated goat anti-mouse IgG antibodies (Bethyl, Inc.) diluted at 1:8,000 in 0.5% skim milk in PBS for 1 h at room temperature. After washing three times, the protein was visualized on X-ray film using the ECL Detection Reagents (GE Healthcare) according to the manufacturer's recommendations.

Sandwich enzyme-linked immunosorbent assay (ELISA) for the detection of secreted TgPDCD5. Vero cells (1×10^5) were inoculated with *T. gondii* PLK-WT, PLK-GFP, PLK-TgPDCD5 and PLK-Δ106 (5×10^5) and incubated in the GIT medium (Nihon Pharmaceutical co., LTD, Tokyo, Japan) at 37°C for 24, 48, 72 and 96 h. The culture supernatant at each time point were collected by sequential centrifugations (500 × *g*, 10 min, 20°C and then 8,400 × *g*, 10 min, 20°C) and concentrated with an NANOSEP 3K OMEGA filter (Pall Corporation).

Rabbit anti-rTgPDCD5 polyclonal IgG was purified using Protein A Chromatography Columns according to the manufacturer's instructions (Bio-Rad Laboratories). Protein concentration was measured using a BCA protein assay kit (Thermo Fisher Scientific, Inc.). One microgram of the IgG diluted in a 0.05 M

carbonate buffer (pH 9.6) was used as the capture antibody to coat microtiter plates at 4°C overnight. Blocking was performed with a blocking solution (3% skim milk in PBS [pH 7.2]) at 37°C for 2 h. The plates were incubated at 37°C for 30 min with each concentrated supernatants. After washing 6 times with a washing solution (0.05% Tween 20 in PBS), anti-rTgPDCD5 mouse serum diluted at 1:100 in a blocking solution was added in each well as a detection antibody, then incubated at 37°C for 1 h. After washing 6 times again, the plates were incubated with HRP-conjubgated goat anti-mouse IgG antibodies (Bethyl, Inc.) diluted at 1:2,500 in a blocking solution at 37°C for 1 h. Binding was visualized with a substrate solution (0.3 mg/ml 2,2'-azino-bis-(3-ethlbenz-thiazoline-6-sulfonic acid), 0.1 M citric acid, 0.2 M sodium phosphate, 0.003% H_2O_2). The absorbance at 415 nm was measured by using and MTP-500 microplate reader (Corona Electric, Tokyo, Japan).

Apoptosis assay. J774A.1 cells (2.5×10^5) were inoculated with *T. gondii* PLK-WT, PLK-GFP, PLK-TgPDCD5 and PLK-Δ106 (5×10^5) then incubated with or without 20 unit/ml of IFN-γ (Thermo Fisher Scientific, Inc.) for 24 h at 37°C. Cells were scraped, washed with PBS, re-suspended in PBS, and placed on glass slides. The slides were dried and then fixed with 4% PFA in PBS before use. After washing twice with PBS, the slides were permeabilized with 0.1% Triton X-100 in 0.1% sodium citrate for 2 min on ice. After washing, the slides were incubated with an *In Situ* Cell Death Detection Kit, TMR red (Roche Diagnostics) at 37°C for 1 h to stain apoptotic cells with TUNEL method. After washing twice, the slides were examined using a fluorescence microscope (Nikon). The mean ratio of apoptotic cells was calculated by counting TUNEL-positive cells and total cells in three individual fields from each slide. Each field contained at least three hundred cells. The statistical significance was

calculated with the Student's *t*-test from the mean and S.D. of triplicate samples.

3-3. Results

Construction and characterization of *T. gondii* overexpressing TgPDCD5. The recombinant parasite overexpressing TgPDCD5 with HA tag was established to confirm the effect of high level TgPDCD5 on the apoptosis in host cells. Two independent clones were isolated from the transfected polyclonal culture, and designated as PLK-TgPDCD5-1 and -2, respectively. The reactivity of the recombinant parasites to the anti-HA monoclonal antibody was confirmed by IFAT (Fig. 8A). The cytosolic localization of TgPDCD5-HA was consistent with that of endogenous TgPDCD5 (Fig. 3A).

Western blot analysis was also performed to confirm the expression of endogenous and induced TgPDCD5. The blot probed with anti-rTgPDCD5 sera indicated that PLK-TgPDCD5 clones expressed additional band in a slightly larger size (15.5 kDa) than that of endogenous protein (Fig. 8B). The expression of induced TgPDCD5-HA was confirmed also in the anti-HA antibody-probed blot, although the existence of two bands for each lanes was not expected (Fig. 8B). The upper band was consistent with the one observed in the anti-rTgPDCD5 blot as an additional band, while the lower one might be the degraded product. A recombinant parasite overexpressing Δ106-122 with HA tag, which lacks the HSPG-binding motif, was also established and designated as PLK-Δ106. The expression of the native and induced TgPDCD5 was confirmed in the IFAT and Western blotting as well as the PLK-TgPDCD5.

TgPDCD5 detected in the supernatant of PLK-TgPDCD5-infected cells.

The secretion of TgPDCD5 by the extracellular parasite and its dependency on incubation time was confirmed in the chapter 1. To clarify its secretion/release from the intracellular parasite and/or parasitized cells, the supernatant of parasite-infected culture were tested whether TgPDCD5 can be detected by using the sandwich ELISA system. The protein detected in PLK-WT-infected Vero cells increased according to the time extension up to 0.081 ± 0.016 OD415 nm at 96 h post infection (Fig. 9). Since the overproduction of full-length/truncated TgPDCD5 in addition to the native protein was confirmed in the Western blot analysis (Fig. 8B), I tested the supernatants from the cells infected with the recombinant parasites. Significantly higher levels of TgPDCD5 were detected in the culture media of cells infected with PLK-TgPDCD5-1 (0.150 ± 0.012, $P < 0.01$), PLK-TgPDCD5-2 (0.184 ± 0.015, $P < 0.01$), and PLK-Δ106 (0.194 ± 0.028, $P < 0.01$) compared to the PLK-WT (0.081 ± 0.016) and PLK-GFP (0.103 ± 0.027) (Fig. 9).

Evaluation of PLK-TgPDCD5 on host cell apoptosis in the presence with IFN-γ. The effect of differing concentration of rTgPDCD5 on host cell apoptosis was confirmed in the chapter 2, indicated the dose-dependency of the pro-apoptotic effect (Fig. 5). In this experiment, PLK-TgPDCD5-1, -2, and PLK-Δ106, PLK-WT, and PLK-GFP parasites were inoculated to the J774A.1 cells and treated with IFN-γ. The rates of apoptotic cells observed in PLK-TgPDCD5-1- and PLK-TgPDCD5-2-infected groups were 24.79 ± 0.83% and 24.54 ± 0.75%, respectively, showing significantly higher levels than those in PLK-Δ106 (12.07 ± 1.10%)-, PLK-WT (9.28 ± 0.05%)-, and PLK-GFP (8.61 ± 0.84%)-infected groups (Fig. 10). This result strongly suggested that the increased amount of TgPDCD5 protein was linked to the high rate of host cell apoptosis.

3-4. Discussion

The sandwich ELISA successfully detected TgPDCD5 protein in the culture medium, showing gradually increased level according to the incubation time which also reflects parasite proliferation. In the measurement of TgPDCD5 from PLK-TgPDCD5-infected culture medium, overproduced TgPDCD5 seemed not to be confined in the parasite, but secreted to the medium together with the native protein (Fig. 9). Even at 24 h post infection, the medium from PLK-TgPDCD5-infected culture contained significantly higher TgPDCD5 than control parasite-treated cells. This may be the case that the protein was derived from extracellular parasite before invading the host cells, although it may contain not only the actively secreted molecules but also the ones which were passively released from dead parasites. Considering the result of apoptosis assay, TgPDCD5 in the medium which was secreted/released by the parasite would be the cause of apoptosis induction in host cells.

The apoptosis enhancement by recombinant proteins and IFN-γ applied to parasite-infected macrophages showed that the apoptosis rate, to some extent, reflected the amount of TgPDCD5 in the medium (Chapter 2, Table 1). This observation was consistent with the result from the cells treated with the different dose of rTgPDCD5 with etoposide (Chapter 2, Fig. 5). The apoptosis assay using overexpress parasite also theoretically reflected the suitability of dose-dependency (Fig. 10). However, significantly higher induction of apoptosis was only observed in the PLK-TgPDCD5 and not in the PLK-Δ106, suggesting the necessity of HSPG-binding motif as observed in the assay using recombinant proteins (Chapter 2, Fig. 6). This may be due to that the

overproduced Δ106-122 protein, not having the motif which was required for cell penetration, seemed not to be internalized by uninfected cells.

This chapter showed that the parasite-produced TgPDCD5 was active to induce host cell apoptosis, and the effect was dependent on the amount of TgPDCD5 in the medium and cell penetration via HSPG-binding motif.

3-5. Summary

The recombinant parasite which overexpresses TgPDCD5 was established to evaluate whether the endogenous TgPDCD5 is really involved in the macrophage apoptosis during *T. gondii* infection. An IFAT study indicated that the overproduced TgPDCD5 with HA tag localized in the cytosol, which was consistent with the localization of the endogenous TgPDCD5. The induced TgPDCD5-HA was recognized as the 15.5 kDa band by Western blot analysis, showing the successful overexpression of TgPDCD5. Secretion/release of TgPDCD5 by the parasite was also up-regulated in a time-dependent manner, which is also reflecting the overproduction. The apoptosis caused by the parasite infection and IFN-γ was significantly up-regulated by the overexpression of TgPDCD5. This result suggests that the endogenous TgPDCD5 plays a role in the macrophage apoptosis during parasite infection.

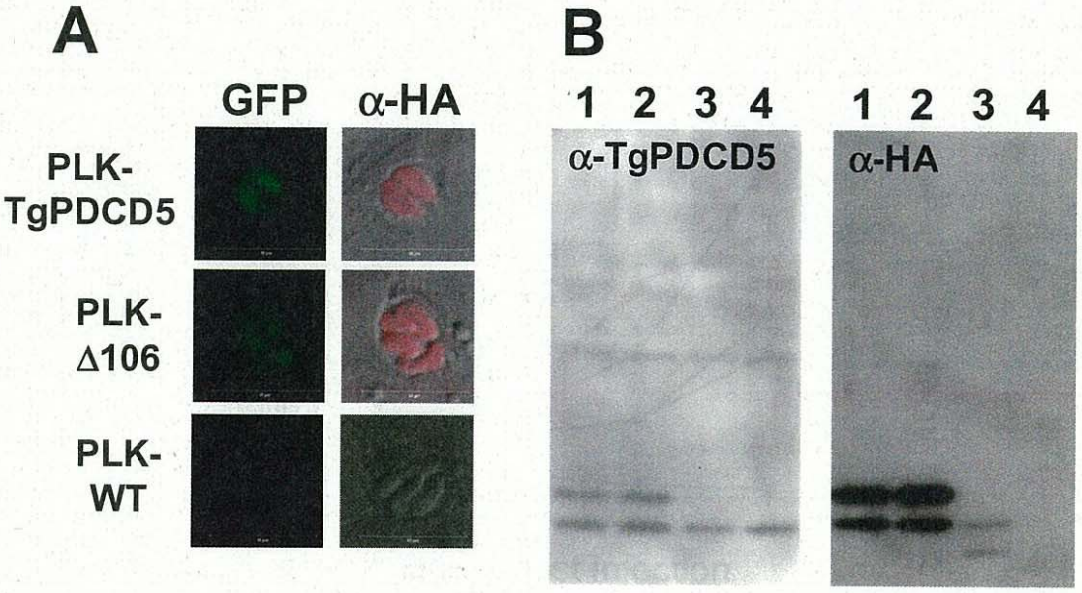

Fig. 8. Expression of TgPDCD5 with HA tag in PLK-TgPDCD5 and PLK-Δ106.
(A) IFAT images of PLK-TgPDCD5 and PLK-Δ106. *T. gondii*-infected CHO-K1 cells on the coverslips were fixed and immuno-stained with mouse anti-HA monoclonal antibody and Alexa flour 594-conjugated goat anti-mouse IgG. Bar, 10 μm.
(B) Western blot analysis of PLK-TgPDCD5 and PLK-Δ106. The lysates from recombinant and wild type parasites were electrophoresed in a 15% polyacrylamide gel and transferred onto a nitrocellulose membrane. It was then probed with the mouse anti-rTgPDCD5 antibody (left) or the mouse anti-HA monoclonal antibody (right), followed by HRP-conjugated goat anti-mouse IgG antibody. Lane 1, PLK-TgPDCD5-1; lane 2, PLK-TgPDCD5-2; lane 3, PLK-Δ106; lane 4, PLK-WT.

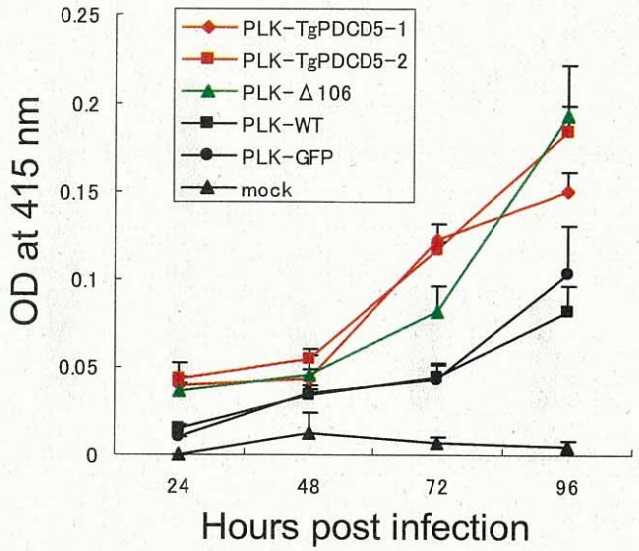

Fig. 9. TgPDCD5 detected in the culture medium of parasite-infected cells.
Vero cells (1×10^5) were inoculated with *T. gondii* PLK-TgPDCD5-1, PLK-TgPDCD5-2, PLK-Δ106, PLK-WT, and PLK-GFP (5×10^5) and incubated in the GIT medium. The culture supernatant at 24, 48, 72 and 96 h post infection were collected and concentrated. The samples were incubated in the 96-well plate coated with rabbit anti-rTgPDCD5 polyclonal IgG, and detected with mouse anti-TgPDCD5 serum and HRP-conjubgated goat anti-mouse IgG antibodies. After reacted with the substrate solution, the absorbance at 415 nm was measured. The line graph indicates the mean and S.D. of triplicate samples. Statistical significance was calculated with the Student's *t*-test.

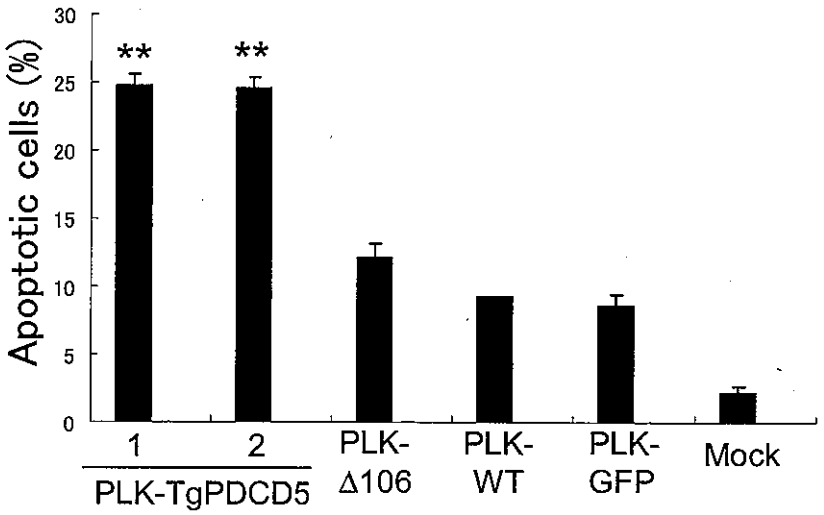

Fig. 10. Evaluation of PLK-TgPDCD5 on host cell apoptosis in the presence with IFN-γ. J774A.1 cells (5×10^5) were inoculated with *T. gondii* PLK-TgPDCD5-1, PLK-TgPDCD5-2, PLK-Δ106, PLK-WT, and PLK-GFP (5×10^5) incubated with 20 unit/ml IFN-γ. After the cells were treated, genomic DNA fragmentation was detected with TUNEL staining, and the ratio of apoptotic cells was determined. The histograms indicate the mean and S.D. of triplicate samples. Statistical significance was calculated with the Student's *t*-test. ** $P < 0.01$.

General discussion

A variety of factors have been described that may contribute to the *T. gondii*'s ability to establish and maintain a persistent infection in its immunocompetent host. Accumulating evidence indicates that this also includes alterations of apoptosis in distinct host cell populations [Heussler et al., 2001; Lüder et al., 2001]. This is not surprising because apoptosis is known to play a critical role in the regulation of the immune response [Opferman and Korsmeyer, 2003], as an effector mechanism of natural killer (NK) cells and cytotoxic T lymphocytes to eliminate infected target cells [Lieberman, 2003], and as an innate response of cells after infection by intracellular pathogens [Williams et al., 1994]. More interestingly, *T. gondii* both promotes and inhibits apoptosis. Inhibition of host cell apoptosis may allow undisturbed intracellular development, thereby facilitating parasite survival. Increased apoptosis of immune cells after infection, on the other hand, is thought to partially disturb the host immune responses against *T. gondii,* leading to immune evasion.

Both CD8+ and CD4+ T lymphocytes are thought to be critical importance in mediating host protection against toxoplasmosis [Gazzinelli et al., 1991]. During primary infection, however, parasite-induced production of down-regulatory cytokines such as IL-10 [Khan et al., 1995], transforming growth factor-beta (TGF-β) [Bermudez et al., 1993], and IFN-γ [Channon and Kasper, 1996] as well as reactive nitrogen intermediates [Candolfi et al., 1994] has been implicated in the immunosuppression. Furthermore *T. gondii* actively down-regulates expression of MHC class I and class II molecules in murine macrophages/monocyte, which may directly inhibit the antigen presenting capacity [Lüder et al., 1998; Lang et al., 2006]. Apoptosis caused by

parasite infection in splenocytes, including CD4+ and CD8+ lymphocytes, B lymphocytes, NK cells, and granulocytes, and in Peyer's patch T cells also may contribute to the suppressed immune response [Liesenfeld et al., 1997; Gavrilescu and Denkers, 2003]. Likewise, the apoptosis observed in macrophages is also considered to be one of the immune evasion mechanisms of the parasite, by suppression of antigen presenting cells [Hisaeda et al., 1997; Nishikawa et al., 2007]. Hence, inhibition of both antigen-presentation by the antigen-presenting cells and antigen-recognition by lymphocytes may be implicated in the reduced immune response caused by *T. gondii*-infection.

Although rTgPDCD5 itself cannot be an apoptosis initiator, once triggered by another factor, for example etoposide, or supported by IFN-γ, it works as a pro-apoptotic molecule. The concentration of recombinant protein which showed apoptosis enhancement was micro-molar order while only pico-molar levels of native TgPDCD5 was detected in the parasitized cell culture (data not shown), suggesting the presence of other factor(s) involved in parasite-induced apoptosis. Further study to identify such yet unknown factor(s) will be necessary. The effect of TgPDCD5 on other kinds of immune cells and its involvement in the apoptosis observed in various tissues during parasite-infection should be also addressed.

HSPGs are ubiquitous molecules found on the surface of most mammalian cells. HSPG-dependent binding or internalization of bioactive molecules is involved in various kinds of biological aspects, such as the regulation of growth factor or cytokine signaling, coagulation factor activity, microbe-host interaction, and lipoprotein metabolism [Shieh et al., 1992; Paul, 1995; Fuki et al., 1997; Van Putten and Mahley and Ji, 1997; Dehio et al., 1998; Colin et al., 1999; Shukla et al., 1999]. Many bacteria,

viruses and parasites have been shown to exploit cell-surface proteoglycans as adhesion receptors. In protozoan parasites, the *Trypanosoma cruzi* and *Leishmania donovani* attachment to cells is mediated by HSPGs [Ortega-Barria and Pereira, 1991; Butcher et al., 1992]. In the case of *P. falciparum*, infection of hepatocytes is initiated by the recognition of HSPGs [Frevert et al., 1993]. The involvement of circumsporozoite protein and thrombospondin-related anonymous protein, both of which have the highly basic region consistent with HSPG-binding motifs, was confirmed [Sinnis et al., 1996; Pinzon-Ortiz et al., 2001; Rathore et al., 2001; Matuschewski et al.,2002; Sinnis et al., 2002; Akhouri et al., 2004]. The parasite attachment to the host cell proteoglycans was observed also in *T. gondii* and was necessary for the parasite invasion [Ortega-Barria and Boothroyd, 1998; Carruthers et al., 1999]. MIC2 and SAG3 proteins were identified as the parasite ligands for the attachment, which also had the HSPG-binding motifs [Carruthers et al., 1999; Jacquet et al., 2001]. In contrary to MIC2 and SAG3 which play roles in the parasite attachment to the cells, TgPDCD5 penetrates the host cells via HSPG and exerts its function within the cells to facilitate apoptosis. Thus this molecule gave us a new insight of interaction between host cells and parasite.

Several reports on human PDCD5 have provided insights into where and how TgPDCD5 functions. Translocation of endogenous or artificially added recombinant PDCD5 from the cytosol to the nucleus during apoptosis [Chen et al., 2001; Wang et al., 2006] may suggest a role of this molecule in the nucleus. Several reports on apoptosis have focused on distinctive nuclear morphological changes in the apoptotic process [Hengartner, 2000; Robertson et al., 2000]. Even though I did not find direct evidence for the nuclear localization of the molecule, TgPDCD5 may have functions

related to nuclear changes and may facilitate host-cell apoptosis.

Another possible explanation for the function of this molecule is alteration of host anti-/pro-apoptotic molecules. In the case of apoptosis in T lymphocytes of *T. gondii*-infected mice, the up-regulation of Fas and FasL was reported, which was regulated by the secretion of proinflammatory cytokines, IL-12 and IFN-γ, and may be counterbalanced by activation of NF-κB_2 [Caamano et al., 2000]. On the other hand, human dendritic cells infected with *T. gondii* have been shown to induce T lymphocyte apoptosis in a contact-dependent and Fas-independent manner [Wei et al., 2002]. These reports suggested the possibility that both Fas-dependent and -independent cell death deplete T cells during toxoplasmosis. The apoptosis observed in peritoneal macrophages from infected mice has been shown to be due to NO [Nishikawa et al., 2007]. In the case of macrophages treated with NO donor such as S-nitrosoglutathione *in vitro*, apoptosis-signaling kinase 1 was reported to be activated, which then induces programmed cells death via MAP kinase-dependent pathway [Sumbayev, 2003; Yasinska et al., 2004]. This study did not address the point in which TgPDCD5 functions. Further study to elucidate the whole mechanism by which TgPDCD5 stimulates apoptosis in *T. gondii*-infected cells or animals will be required.

In conclusion, this is the first report to identify the parasite molecule that exerts a pro-apoptotic effect on host cells. Further study for detailed functions of this molecule and other yet un-identified parasite molecules will lead us to elucidate the whole mechanisms of parasite-mediated apoptosis and its interaction with pathogenesis.

General summary

Although parasite-infected host cells become resistant to apoptosis, uninfected bystander cells undergo apoptosis during *Toxoplasma gondii* infection. The *Programmed Cell Death 5* (*TgPDCD5*) gene, a homologue of the human apoptosis-related molecule, was cloned from a *T. gondii* full-length cDNA database and subsequently characterized. The native TgPDCD5 was located in the cytosol and also detected in the secreted fraction. Immuno-electron microscopic analysis showed TgPDCD5 was primarily located close to the rhoptries or vesicle-like structures near the surface membrane of the parasite. Studies using recombinant TgPDCD5 (rTgPDCD5) demonstrated that host cells internalize the molecule in a heparan sulfate proteoglycan-binding motif-dependent manner. Furthermore, the addition of rTgPDCD5 to culture medium resulted in the enhancement of host-cell apoptosis triggered by etoposide in macrophage cell line J774A.1 and leukemic cell line HL-60 cells. Additionally, rTgPDCD5 induced apoptosis in J774A.1 cells in the presence of IFN-γ. The study using recombinant parasite overexpressing TgPDCD5 clearly indicated the involvement of this protein in the apoptosis observed in macrophages during *T. gondii* infection. This report is the first to identify a parasitic molecule of *T. gondii* that has a pro-apoptotic effect on host cells.

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