Overproduction of the Pro-Apoptotic Molecule, Programmed Cell Death 5, in *Toxoplasma gondii* Leads to Increased Apoptosis of Host Macrophages

Hiroshi BANNAI1), Yoshifumi NISHIKAWA1)*, Hany Mohamed IBRAHIM1), Kyoko YAMADA1), Osamu KAWASE1), Jun-ichi WATANABE2), Chihiro SUGIMOTO3) and Xuenan XUAN1)

1) National Research Center for Protozoan Diseases, Obihiro University of Agriculture and Veterinary Medicine, Inada-cho, Obihiro, Hokkaido 080–8555, 2) The Institute of Medical Science, The University of Tokyo, Shirokanedai, Minato-ku, Tokyo 108–8639 and 3) Research Center for Zoonosis Control, Hokkaido University, Chuou-ku, Sapporo 060–0818, Japan

(Received 22 March 2009/Accepted 17 May 2009)

**ABSTRACT.** We established a recombinant strain of *Toxoplasma gondii* that overexpressed programmed cell death 5 (TgPDCD5), in order to evaluate the role of endogenous TgPDCD5 in macrophage apoptosis during *T. gondii* infection. Immunofluorescence microscopy revealed that overproduced TgPDCD5 with a hemagglutinin tag was localized in the cytosol, which was consistent with the localization of endogenous TgPDCD5. The induced TgPDCD5-HA was recognized as an additional band by Western blot analysis, indicating successful overexpression of TgPDCD5. Secretion and release of TgPDCD5 by the parasite was also up-regulated in a time-dependent manner, which reflected its overproduction. Apoptosis due to parasite infection and interferon-gamma treatment was significantly up-regulated by the overexpression of TgPDCD5. These results suggest that endogenous TgPDCD5 plays a role in macrophage apoptosis during *T. gondii* infection.

**KEY WORDS:** apoptosis, overexpression, PDCD5, *Toxoplasma gondii.*

---

*Toxoplasma gondii* is an obligate intracellular parasite that can infect virtually all species of warm-blooded animals, including humans, worldwide. Domestic cats and other felines are the definitive hosts, while non-feline hosts act as intermediate hosts [3]. *T. gondii* infection is usually asymptomatic in immunocompetent hosts, and severe cases occur only rarely. However, it remains a serious threat to human health due to its high prevalence, which is typically 10–30% in most populations, but sometimes even higher [33]. In addition to causing life-threatening encephalitis in immuno-compromised hosts such as AIDS patients, *T. gondii* can also cause miscarriage, stillbirth, or fatal birth defects in humans if contracted during pregnancy [23, 29].

Acute infection of both humans and mice with *T. gondii* induces as state of transient immunosuppression, characterized by reduced antibody and T lymphocyte responses [22, 32, 38, 39]. In addition, the apoptosis of T lymphocytes triggered by *T. gondii* has been thought to restrict the immune response to the parasite [14, 17, 36]. Indeed, high levels of apoptosis in splenocytes have been associated with unrestricted parasite multiplication leading to high parasite burdens in various host tissues [6, 24]. Apoptosis of peritoneal macrophages has also been reported in infected mice [11, 25]. Apoptosis in macrophages was also confirmed in an in vitro study, which showed that *T. gondii* infection of J774A.1 mouse macrophage cells treated with interferon-gamma (IFN-γ) resulted in activation of inducible nitric oxide synthase, and that the consequent production of nitric oxide facilitated apoptosis [25]. This immune cell apoptosis is thought to lead to immunosuppression, and so contribute to parasite survival in the host.

*T. gondii* programmed cell death 5 (TgPDCD5) was identified as a homologue of the human apoptosis-related molecule PDCD5 [1, 2, 18, 30, 34, 35] and the *Plasmodium falciparum* apoptosis-related protein [9]. We reported that recombinant TgPDCD5 (rTgPDCD5) exerted an apoptosis-enhancing effect on host cells in the presence of the topoisomerase II inhibitor, etoposide, or in cooperation with IFN-γ [1]. The cell-penetration activity of rTgPDCD5 via a heparan-sulfate proteoglycan (HSPG)-binding motif was confirmed to be highly associated with its pro-apoptotic effect, as observed in the human PDCD5 protein [1, 34]. Endogenous TgPDCD5 is a secreted protein, despite having no signal peptide, suggesting that its secretion occurs not via the typical pathway seen in microneme proteins or rhoptry proteins, but via an unknown mechanism. This is a common feature of previously described translocatory proteins with highly basic regions such as human immunodeficiency virus Tat [5, 8], *Drosophila* antennapedia [12], and herpes simplex virus VP22 [4]. These proteins are known to penetrate into the cells via HSPG on the surface membrane, and this fact can be applied for the development of drug delivery tools.

In order to clarify the role of endogenous TgPDCD5 in host cell apoptosis during *T. gondii* infection, we established a recombinant parasite that overexpressed TgPDCD5. We aimed to clarify the role of endogenous TgPDCD5 in host macrophages and establish whether or not it showed similar apoptosis-enhancing activity to the recombinant protein.
MATERIALS AND METHODS

Cell lines: The mammalian cell lines used in this study were Chinese hamster ovary epithelial cells (CHO-K1, American Type Culture Collection [ATCC] CCL-61), mouse macrophages (J774A.1, ATCC TIB-67), and monkey kidney adherent fibroblasts (Vero, ATCC CCL-81). Cells were maintained as described in the ATCC instructions. T. gondii isolates used were PLK wild-type strain (PLK-WT), a recombinant PLK strain that expressed green fluorescent protein (GFP) (PLK-GFP) [27], and recombinant PLK strains that overexpressed 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.

Production of anti-GST-TgPDCD5 serum and purification of IgG: rTgPDCD5 fused with glutathione S-transferase (GST) was expressed in Escherichia coli, and anti-GST-TgPDCD5 serum from mice and a rabbit were produced as described previously [1]. 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.

Construction of transfer vector: The plasmid for overexpression in T. gondii, pDMG was constructed to express GFP, dihydrofolate reductase-thymidylate synthase (DHFR-TS), and the target protein [26]. The DNA fragment for full-length TgPDCD5 was amplified by polymerase chain reaction from the full-length cDNA clone XTG05779 of Full-Toxo [13] using the primers 5'-ATC CAT GGA TGC AGC CTG AAG AAT TCG CC-3' and 5'-CAT GGA TGC AGC CTG AAG AAT TCG CC-3' and 5'-TAG CTA GCC GCA GAC GCC GC-3' was used. The resulting products were digested with NcoI and Nhel and inserted into the same sites of pHXNTPHA (kindly provided by K.A. Joiner, Yale University), to produce pHXNTP-TgPDCD5 (or Δ106–122)-hemagglutinin (HA). TgPDCD5 (or Δ106–122) with a HA tag (YPYDVPDYA) was obtained from pHXNTP-TgPDCD5 (or Δ106–122)-HA by NcoI and BglII digestion. This fragment was blunt ended by Klenow fragment and inserted into the EcoRV site of pDMG; pDMG-TgPDCD5 or Δ106-122-HA by NcoI and BglII.

Transfection and selection of T. gondii-overexpressing TgPDCD5 or Δ106–122: Electroporation of tachyzoites was performed as described previously [31]. In brief, 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 W using a Gene Pulser II (Bio-Rad Laboratories, Hercules, U.S.A.). After transfection, tachyzoites were allowed to infect Vero cells in drug-free culture medium for 18 hr to permit the phenotypic expression of the DHFR-TS gene and GFP selectable markers, after which pyrimethamine was added to a final concentration of 1 μM. Polyclonal transfected, pyrimethamine-resistant tachyzoite cultures were subjected to plaque purification. The cultures were passaged three times in 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.

Indirect fluorescent antibody test (IFAT): Confluent CHO-K1 cells on coverslips were inoculated with PLK-TgPDCD5 or PLK-Δ106. The coverslips were collected at 24 hr post-parasite inoculation, washed, fixed and then subjected to IFAT as described previously [1]. Briefly, the coverslips were treated with anti-HA monoclonal antibody (Covance Research Products, Inc., Berkeley, U.S.A.) as primary antibody diluted at 1:500 in 3% bovine serum albumin (BSA) in phosphate-buffered saline (PBS) containing 1 mM CaCl_2 and MgCl_2 (PBS++) for 1 hr at room temperature. After washing three times with PBS++, the coverslips were incubated with Alexa Fluor 594-conjugated goat anti-mouse IgG (Invitrogen, Carlsbad, U.S.A.) as secondary antibody diluted at 1:600 in 3% BSA in PBS++ for 1 hr at room temperature and then washed again. For the control staining of primary and secondary antibodies were 1:100 and 1:600, respectively. The coverslips were placed on a glass slide coated with Mowiol (EMD Biosciences, Inc., La Jolla, U.S.A.). The slides were examined by confocal laser scanning microscopy (TCS-NT, Leica Microsystems GmbH, Wetzlar, Germany).

Western blot analysis of PLK-TgPDCD5 and PLK-Δ106: The parasite lysate was prepared according to the method described previously [1]. Briefly, 1 × 10^8 tachyzoites of PLK-WT, PLK-TgPDCD5 or PLK-Δ106 strains were harvested, lysed and sonicated. Protein samples were electrophoresed in each lane of a 15% polyacrylamide gel and transferred onto a nitrocellulose membrane (Whatman GmbH, Dassel, Germany). After washing twice with a washing solution, the membrane was incubated with 5% skimmed milk in PBS for 30 min at room temperature. After two further washes, the membrane was incubated with anti-TgPDCD5 mouse serum, anti-HA mouse monoclonal antibody, or anti-SAG1 mouse monoclonal antibody (Advanced Immunochemical, Inc., Long Beach, U.S.A.) for 1 hr at room temperature. The dilutions of primary and secondary antibodies were 1:100 for anti-TgPDCD5 mouse serum, 1:1,000 for anti-HA antibody, and 1:500 for anti-SAG1 antibody. After washing three times, the membranes were incubated with horseshadish peroxidase (HRP)-conjugated goat anti-mouse IgG antibodies (Bethyl, Inc., Montgomery, U.S.A.) diluted at 1:8,000 in 0.5% skimmed milk in PBS for 1 hr at room temperature. After washing three times, the proteins were visualized by enhanced chemiluminescence reagent (Amersham Biosciences, Inc., Arlington Heights, Ill.).
were visualized on X-ray film using the ECL Detection Reagents (GE Healthcare UK Ltd., Buckinghamshire, UK) according to the manufacturer’s recommendations.

Sandwich enzyme-linked immunosorbent assay (ELISA) for the detection of secreted TgPDCD5: In order to detect secreted TgPDCD5 from extracellular parasites, purified T. gondii tachyzoites of PLK-WT, PLK-GFP, PLK-TgPDCD5 and PLK-Δ106 (5 × 10⁶) were incubated in 100 μl of GIT medium (Nihon Pharmaceutical Co., Ltd., Tokyo, Japan) at 37°C for 15 or 30 min. The culture supernatant at each time point was collected by sequential centrifugation (500 × g for 10 min at 4°C, then 8,400 × g for 10 min at 4°C), and subjected to sandwich ELISA without condensation.

Rabbit anti-rTgPDCD5 polyclonal immunoglobulin-G (IgG) was purified using Protein A chromatography columns according to the manufacturer’s instructions (Bio-Rad Laboratories). Protein concentrations were measured using a BCA protein assay kit (Thermo Fisher Scientific, Inc., Rockford, U.S.A.). 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% skimmed milk in PBS, pH 7.2) at 37°C for 2 hr. The plates were incubated at 37°C for 30 min with each concentrated supernatant. After washing six 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 to each well as a detection antibody. After a further 6 washes, the plates were incubated with HRP-conjugated goat anti-mouse IgG antibodies (Bethyl, Inc.) diluted at 1:2,500 in a blocking solution. Binding was visualized using a substrate solution (0.3 mg/ml 2,2’-azino-bis-(3-ethylbenz-thiazoline-6-sulfonic acid), 0.1 M citric acid, 0.2 M sodium phosphate, 0.003% H₂O₂). The absorbance at 415 nm was measured using a MTP-500 microplate reader (Corona Electric, Tokyo, Japan). The TgPDCD5 concentration of each sample was calculated by standardization with rTgPDCD5.

Apoptosis assay: J774A.1 cells (2.5 × 10⁵) were inoculated with T. gondii PLK-WT, PLK-GFP, PLK-TgPDCD5 or PLK-Δ106 (5 × 10⁵) and then incubated with or without 20 U/ml of IFN-γ (Thermo Fisher Scientific, Inc.) for 24 hr 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, permeabilized and then stained with an In Situ Cell Death Detection Kit, TMR red (Roche Diagnostics GmbH, Mannheim, Germany) to visualize apoptotic cells using the terminal deoxynucleotidyltransferase-mediated dUTP-biotin nick-end labeling (TUNEL) method, as described previously [1]. The slides were examined using a fluorescence microscope (Nikon, Tokyo, Japan). The mean ratio of apoptotic cells was calculated as the number of TUNEL-positive cells divided by the total number of cells × 100, in three individual fields from each sample. Each field contained at least 300 cells. Statistical significance was calculated using Student’s t-test based on the mean and S.D. of triplicate samples.

RESULTS

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

Western blot analysis was performed to confirm the expression of endogenous and induced TgPDCD5. An anti-SAG1 antibody was used as a control to confirm that each lane contained the same amount of parasite lysate. Western blotting with anti-rTgPDCD5 sera indicated that PLK-TgPDCD5 clones expressed additional bands of a slightly larger size (15.5 kDa) than that of the endogenous protein (14.5 kDa) (Fig. 1B). The expression of induced TgPDCD5-HA was also confirmed in the anti-HA antibody-treated blot, although the existence of two bands in each lane was not expected (Fig. 1B). The upper band was consistent with that observed as the additional band in the anti-rTgPDCD5 blot while the lower one possibly represented a degraded product. A recombinant parasite overexpressing Δ106–122-HA, which lacks the HSPG-binding motif, was also established and designated as PLK-Δ106. The expression of induced Δ106–122-HA was confirmed by IFAT as in PLK-TgPDCD5 (Fig. 1A). The single band (14.5 kDa) seen in the PLK-Δ106 lysate treated with anti-rTgPDCD5 serum by Western blot may be due to the similar sizes of native TgPDCD5 and Δ106–122-HA (Fig. 1B). Two bands were present in the anti-HA antibody-treated blot, as observed for PLK-TgPDCD5. The upper band was consistent with the predicted size of Δ106–122-HA (14.5 kDa), while the lower one possibly represented a degraded product. An HA tag was fused with C-terminus of TgPDCD5 or Δ106–102. Therefore, lower band detected by anti-HA antibody may be a product cleaved about 9 amino acids from the N-terminus of the intact TgPDCD5 or Δ106–102. However, no domain was predicted to be the signal sequence in TgPDCD5 while this molecule was secreted protein [1]. Since only one band was detected in PLK-WT using anti-TgPDCD5 antibody, degraded products seen using anti-HA antibody might be artifact.

Increased levels of TgPDCD5 secreted/released into the supernatant of PLK-TgPDCD5 and parasitized cells: The secretion of TgPDCD5 by the extracellular parasite and its dependency on incubation time was confirmed in our previous study [1]. The overproduction of full-length and truncated TgPDCD5, in addition to the native protein, was confirmed by Western blot analysis (Fig. 1B), and the secretion of TgPDCD5 from the recombinant parasites was measured using a sandwich ELISA. The secretion of TgPDCD5 from PLK-WT was slightly increased to 126.1 ± 12.9 pg/ml
However, PLK-TgPDCD5–1 (246.1 ± 20.1 pg/ml, P<0.01), PLK-TgPDCD5–2 (228.6 ± 22.0 pg/ml, P<0.01), and PLK-Δ106 (194.9 ± 32.0 pg/ml, P<0.05) showed significantly higher levels of TgPDCD5.

**Evaluation of the effects of PLK-TgPDCD5 on macrophage apoptosis in the presence of IFN-γ:** The effect of different concentration of rTgPDCD5 on host cell apoptosis has been confirmed in our previous study, indicating dose-dependency of the pro-apoptotic effect [1]. In this experiment, PLK-TgPDCD5–1, PLK-TgPDCD5–2, PLK-Δ106, PLK-WT, and PLK-GFP parasites were inoculated into J774A.1 cells and treated with IFN-γ. The ratios of apoptotic cells observed in the PLK-TgPDCD5–1- and PLK-TgPDCD5–2-infected groups were 23.71 ± 1.11% (P<0.01) and 23.40 ± 2.06% (P<0.01), respectively. These were significantly higher than those in the PLK-Δ106- (9.44 ± 1.07%), PLK-WT- (9.45 ± 1.02%), and PLK-GFP-infected (9.38 ± 0.58%) groups (Fig. 3). This result strongly suggests that parasite-expressed TgPDCD5 was active in apoptosis induction, and that an increased level of TgPDCD5 protein was linked to the high rate of host cell apoptosis.

**DISCUSSION**

Many factors have been suggested to contribute to *T. gondii*’s ability to establish and maintain a persistent infection in immunocompetent hosts. Several reports have indicated that this includes alterations in apoptosis in distinct host cell populations [10, 20]. This is not surprising because apoptosis is known to play a critical role in the regulation of the immune response [28], as an effector mechanism whereby, natural killer (NK) cells and cytotoxic T lymphocytes eliminate infected cells [16], and as an innate response of cells after infection by intracellular pathogens [37]. More interestingly, *T. gondii* both promotes and inhibits apoptosis [19]. The 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 to *T. gondii*, leading to immune evasion.

*T. gondii* actively down-regulates the expression of major histocompatibility complex class I and class II molecules in murine macrophages and monocytes, which may directly inhibit their antigen-presenting capacity [15, 21]. 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, may also contribute to suppression of the immune response [7, 17]. Likewise, the apoptosis observed in macrophages is also considered to be
T. GONDII PDCD5 INDUCES APOPTOSIS IN HOST CELLS

a mechanism of immune evasion adopted by the parasite, through suppression of antigen presenting cells [11, 25]. 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.

In our previous study, apoptosis enhancement of parasite-infected macrophages by recombinant proteins and IFN-γ showed that the rate of apoptosis reflected the amount of TgPDCD5 in the medium [1]. This observation was consistent with the results from cells treated with different doses of rTgPDCD5 and with etoposide [1]. The apoptosis assay using the overexpressing parasite also reflected TgPDCD5 dose-dependency. However, significantly increased apoptosis was only observed after PLK-TgPDCD5 infection, but not after PLK-Δ106 infection, suggesting the necessity for the HSPG-binding motif, as observed in the assay using recombinant proteins [1]. This may be due to the fact that the overproduced Δ106–122 protein, lacking the motif required for cell penetration, was not internalized by uninfected cells.

It was also notable that only pico-molar levels of native TgPDCD5 were detected in the culture supernatant at 24 hr after the infection. The concentration of secreted TgPDCD5 in the PLK-TgPDCD5-1-, PLK-TgPDCD5–2- and PLK-Δ106-infected groups were 4.00 ± 0.46 pg/ml (P<0.01), 4.37 ± 0.91 pg/ml (P<0.01) and 3.67 ± 0.42 pg/ml (P<0.01), respectively. Since the levels of apoptotic cells observed in the PLK-TgPDCD5-1- and PLK-TgPDCD5–2-infected groups were significantly higher than those in the PLK-WT- and PLK-GFP-infected groups, supernatant of parasite cultures containing pico-molar level of native TgPDCD5 can enhance host-cell apoptosis. However, the level of recombinant protein that produced apoptosis enhancement in the previous study was in the micro-molar order. This might be due to the difference in expression origins; it is possible that rTgPDCD5 expressed in E. coli might possess a lower apoptosis-enhancing activity than parasite-expressed native protein. Alternatively, the pro-apoptotic effect of this molecule alone may not be strong enough to induce apoptosis; it may require other, additional factors. Further studies are needed to identify such as yet unknown factor(s). The effects 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 in future studies.

A sandwich ELISA successfully detected a time-dependent increase (after 15 min) in TgPDCD5 protein levels in the culture medium of free parasites. We suggested that TgPDCD5 secreted from the extracellular tachyzoites was internalized by host cells before the invasion and by the uninfected host cells. Interestingly, treatment of J774A.1 cells with rTgPDCD5 protein and IFN-γ showed no...
enhancement of host-cell apoptosis in \textit{T. gondii}-infected cells while apoptosis was enhanced in the uninfected cells (1). Since \textit{T. gondii}-infected host cells are resistant against apoptosis induction by interference with the caspase cascade, increased expression of antiapoptotic molecules by infected host cells, and a decreased activity of the poly(ADP-ribose) polymerase (19), effects of TgPDCD5 might be cancelled in the infected cells. In conclusion, endogenous TgPDCD5 protein was active in enhancing host cell apoptosis, as previously reported for recombinant TgPDCD5. The involvement of the HSPG-binding motif was also confirmed, suggesting that penetration of this molecule into the host cells is essential for its activity. However, the mechanism of TgPDCD5 function remains unclear. Further studies are required to elucidate the mechanisms whereby TgPDCD5 stimulates apoptosis in \textit{T. gondii}-infected cells or animals.

ACKNOWLEDGMENTS. We thank Dr. K.A. Joiner (Yale Univ.) for providing the plasmid vector pHXNTPHA. This study was supported by Grants-in-Aid for Scientific Research on Priority Areas (19041008), the Ministry of Education, Culture, Sports, Science and Technology of Japan. The first author has been supported by Research Fellowships of the Japan Society for the Promotion of Science for Young Scientists (19–21).

REFERENCES


24. Mordue, D. G., Monroy, F., La Regina, M., Dinarello, C. A.


