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▣ BRIEF COMMUNICATION

# Influence of 120 kDa Pyruvate:Ferredoxin Oxidoreductase on Pathogenicity of *Trichomonas vaginalis*

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**Abstract:** *Trichomonas vaginalis* is a flagellate protozoan parasite and commonly infected the lower genital tract in women and men. Iron is a known nutrient for growth of various pathogens, and also reported to be involved in establishment of trichomoniasis. However, the exact mechanism was not clarified. In this study, the author investigated whether the 120 kDa protein of *T. vaginalis* may be involved in pathogenicity of trichomonads. Antibodies against 120 kDa protein of *T. vaginalis*, which was identified as pyruvate:ferredoxin oxidoreductase (PFOR) by peptide analysis of MALDI-TOF-MS, were prepared in rabbits. Pretreatment of *T. vaginalis* with anti-120 kDa Ab decreased the proliferation and adherence to vaginal epithelial cells (MS74) of *T. vaginalis*. Subcutaneous tissue abscess in anti-120 kDa Ab-treated *T. vaginalis*-injected mice was smaller in size than that of untreated *T. vaginalis*-infected mice. Collectively, the 120 kDa protein expressed by iron may be involved in proliferation, adhesion to host cells, and abscess formation, thereby may influence on the pathogenicity of *T. vaginalis*.

Key words: *Trichomonas vaginalis*, pyruvate:ferredoxin oxidoreductase, iron, adhesion, abscess formation

*Trichomonas vaginalis* is a flagellated parasitic protozoan known as a common cause of sexually transmitted disease (STD). Iron is an important nutrient for the growth of some pathogens, including trichomonads [1]. Previous studies have demonstrated that concentration of iron in trichomonads medium changes the properties of *T. vaginalis* such as adherence, cytotoxicity, and proteinase activity; however, the exact mechanism is still unclear [2]. Recently, over-expressed 120 kDa protein of *T. vaginalis* cultivated in iron rich condition was identified as pyruvate:ferredoxin oxidoreductase (PFOR) [3]. This PFOR has been shown to involve in adhesion of *T. vaginalis* to HeLa cells [4].

It is not yet clarified whether 120 kDa PFOR may influence on the pathogenicity of *T. vaginalis* clearly. In this study, the author examined the influence of 120 kDa protein of *T. vaginalis* on pathogenic factor such as proliferation, adhesion to vaginal epithelial cells (MS74), and in vivo abscess formation in mice using anti-120 kDa Ab. *T. vaginalis* was cultivated in iron-rich conditions, and then antibodies against 120 kDa protein were made by immunizing with a 120 kDa protein to rabbits. Finally, trophozoites proliferation, host cell adherence, and subcutaneous abscess formation in mice were observed after anti-120 kDa Ab pretreatment.

Four isolates of *T. vaginalis* were used in this experiment. KT4 and KT53 were isolated from the vaginal discharge of Korean females with acute vaginitis. CDC85 was purchased from ATCC (Manassas, Virginia, USA) and T016 isolate were kindly provided by Prof. J. F. Alderete (University of Texas, Health Science Center, Texas, USA). KT53 and CDC85 were shown to have resistance to metronidazole [2]. Trichomonads were subcultured every 24 hr in a 15-ml glass tube containing complex trypticase-yeast extract-maltose medium (TYM) in 5% $CO_2$ in air at 37˚C [5]. Iron-lacked TYM medium was prepared by adding 2,2´-dipyridyl (100 μM), whereas iron-rich TYM medium was prepared by adding ferrous sulfate (200-360 μM).

T016 isolate of *T. vaginalis* was subcultured in TYM, iron-lacked TYM, or iron-rich TYM medium for 72 hr, respectively. Trophozoites ($1 \times 10^6$) were centrifuged, and then washed with PBS twice. The pellet was lysed with 70 μl of lysis buffer which consists of 20 mM Tris-HCl (pH 7.5), 60 mM β-glycerophosphate, 10 mM EDTA, 10 mM $MgCl_2$, 10 mM NaF, 2 mM DTT, 1 mM $Na_3VO_4$, 1 mM APMSF, 1% NP-40, leupeptin (5 μg/ml), 2 mM levamisol, pepstatin A (10 μg/ml), 0.5 mM benzamidine, and 1 tablet of complete mini (protease inhibitor cock-

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tail; Roche, Indianapolis, Indiana, USA). This was incubated in ice for 40 min. Lysed samples were mixed with SDS-PAGE sample buffer and boiled at 100°C for 5 min. The total cellular proteins were subjected to 7.5% SDS–polyacrylamide gel. Protein expression was determined with Coomassie brilliant blue (CBB) staining. Over-expressed 120 kDa protein of *T. vaginalis* in CBB stained gel was collected and treated with trypsin. Protein was analyzed by matrix-assisted laser desorption ionization-time-of-flight peptide mass mapping method (MALDI-TOF-MS) at In2Gen (Seongnam, Korea).

Anti-120 kDa antibodies were prepared in rabbits by repeated immunizations. For the first immunization, rabbits were injected subcutaneously with 120 kDa gel band (100 μg) mixed with Freund's complete adjuvant. The second immunization was performed with 120 kDa gel band (100 μg) mixed with Freund's incomplete adjuvant. The third immunization was done with 120 kDa gel band (100 μg). Each immunization was conducted in 3 week intervals. Rabbit blood was obtained after the third immunization. The rabbit serum containing immunoglobulin G was purified using Proteus protein A kit (Prochem Inc., Acton, Massachusetts, USA) by the manual of the manufacturer. After centrifugation via Proteus protein A kit, purified solutions (1.60 mg/ml) were used as anti-120 kDa Ab.

For the proliferation assay, *T. vaginalis* ($5 \times 10^4$/ml) were seeded in each well of 96-well plate containing 200 μl of TYM medium. Anti-120 kDa Ab (0-800 μg/ml) was added, and then incubated for 48 hr in 5% $CO_2$ in air at 37°C. Trypan blue-stained trophozoites were counted with a hemocytometer. A cytoadherence assay was done according to a previous method [6]. *T. vaginalis* (KT4, KT53, and CDC85) were labeled at a density of $1 \times 10^6$ cells in TYM supplemented with 5 μCi/ml [$^3$H]-thymidine (specific activity 25 Ci/mmol, Amersham, UK) for 18 hr. Vaginal epithelial cells (MS74; $5 \times 10^4$) were seeded in each well of 96-well plate and incubated for 24 hr. Trophozoites ($4 \times 10^5$) labeled with [$^3$H]-thymidine were pretreated with anti-120 kDa Ab (125 μg/ml), then added to monolayer of MS74 cells.

After 1 hr incubation, unbound parasites were removed, and cytoadherence was determined by counting individual wells for radioactivity with a scintillation counter (Packard Instrument Co., Downers Grove, Illinois, USA). Cytoadherence (%) was measured by which cpm of each well was divided by cpm of trophozoites ($4 \times 10^5$) inoculated at the start point. Subcutaneous abscess formation was employed to know about the virulence of *T. vaginalis*. Ten mice were tested per each group. BALB/c mice were anesthetized with ether, and then trophozoites ($1 \times 10^6$) pretreated with anti-120 kDa Ab were injected into the back of mice. After 4 days, mice were sacrificed, and the lesion of abscess and surrounding skin was removed. The size of the abscess was calculated according to following formula: abscess volume = long axis × short axis × π. The results are expressed as the mean ± SEM of 3 independent experiments. The Mann–Whitney U-test was used for statistical analysis, and a *P*-value of $< 0.05$ was considered statistically significant.

Expression of 120 kDa protein was increased by cultivation in iron-rich TYM medium (Fig. 1). Trichomonads cultured in iron-lacked TYM and normal TYM showed weak density of 120 kDa compared with that of iron-rich TYM. The protein band of 120 kDa cultivated in 2 different iron concentrations of iron-rich medium (200 and 360 μM) showed similar density. The peptides from the 120 kDa protein gel band were analyzed by MALDI-TOF-MS (at In2Gen), and the masses of peptides corresponded to peptides mass from the PFOR of *T. vaginalis* in NCBI DB.

Anti-120 kDa Ab (anti-PFOR Ab) was used to know the influence of 120 kDa protein to the trophozoite proliferation, host cell adhesion, and subcutaneous abscess formation. When *T. vaginalis* was co-cultured with anti-120 kDa Ab, proliferation

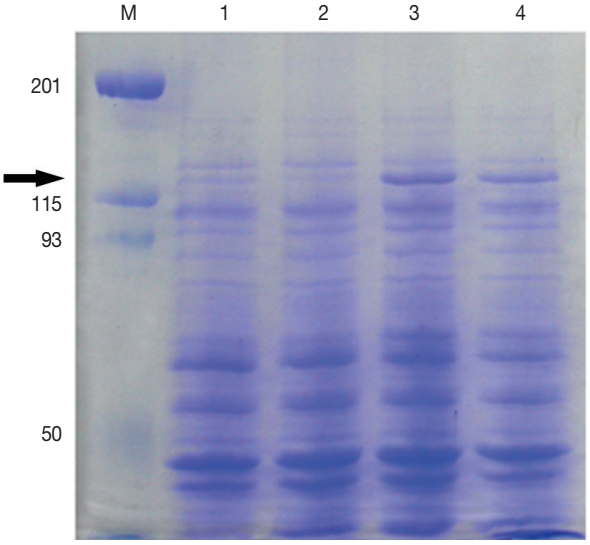


**Fig. 1.** Strong expression of 120 kDa protein in *T. vaginalis* after cultured in iron-rich medium. Trichomonads were incubated in TYM, iron-lacked, or iron-rich TYM medium for 72 hr. Gels were stained with Coomassie brilliant blue stain after SDS-PAGE. Lane 1, trichomonads cultured in iron-lacked TYM; lane 2, trichomonads cultured in normal TYM; lanes 3-4, trichomonads cultured in TYM medium supplemented with 200 μM or 360 μM of ferrous sulfate, respectively. M, standrad size marker; arrow, band of 120 kDa protein.

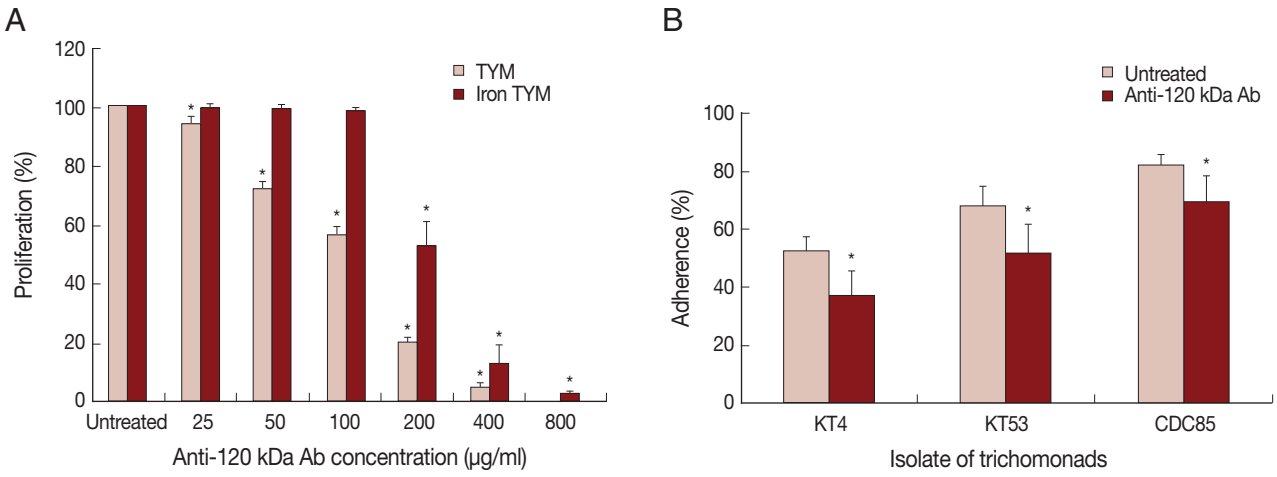


**Fig. 2.** Decrease of proliferation and adherence ability of *T. vaginalis* by anti-120 kDa Ab treatment. (A) T016 isolate of *T. vaginalis* were co-cultured with anti-120 kDa Ab (0-800 μg/ml) in TYM or Iron TYM. Trichomonads were counted with hemocytometer after 48 hr cultivation. (B) Three isolates of trichomonads were labeled with [$^3$H]-thymidine and co-cultured with MS74 cells with or without anti-120 kDa Ab pretreatment. Cpm was measured with β-counter for determination of cell adhesion. *$P<0.05$ compared to untreated control.

**Table 1.** Mouse subcutaneous abscess produced by *T. vaginalis* (T016) pretreated with anti-120 kDa Ab

| Treatment | Abscess ($mm^2$) | No. mice with abscess /No. test mice |
|---|---|---|
| Untreated | 17.4±1.41 | 10/10 |
| Anti-120 kDa Ab | 10.7±1.73[a] | 10/10 |

[a]$P<0.05$ compared to untreated control.

of trophozoites was decreased in a dose-dependent manner. In iron-rich condition, more than 200 μg/ml of anti-120 kDa Ab was required for inhibition of trophozoite growth. However, only 25 μg/ml of anti-120 kDa Ab suppressed the proliferation of trophozoites in normal TYM medium significantly (Fig. 2A). A larger amount of anti-120 kDa Ab may be needed for growth reduction by trichomonads cultivated in iron-rich medium than trophozoites in normal TYM medium. Cytoadherence of 3 *T. vaginalis* isolates to MS74 cells after 120 kDa Ab pretreatment was decreased compared to those of untreated trophozoites (Fig. 2B). Anti-120 kDa Ab pretreatment to *T. vaginalis* also decreased the subcutaneous abscess formation in mice (Table 1).

In this study, the author observed the role of 120 kDa protein (PFOR) of *T. vaginalis* on their proliferation and cytoadherence to vaginal epithelial cells. Iron is an important nutrient for survival of pathogenic protozoa, including *T. vaginalis* [1]. Over-expression of 120 kDa protein was observed in iron-rich conditions. However, the role of the PFOR on pathogenicity of trichomonads remained unclear. In this experiment, the influence of 120 kDa protein on the virulence of *T. vaginalis* was observed by in vivo mouse subcutaneous abscess formation experiment for the first time.

Some protozoa, like *T. vaginalis* and *Entamoeba histolytica*, require high iron concentrations for their growth [7]. Lehker and Alderete [8] reported that iron-rich conditions increased the rate of *T. vaginalis* proliferation and protein synthesis. Pathogens use various methods for iron uptake from the hosts. Microorganisms in insufficient iron conditions often use their required iron from iron-binding proteins [9]. *Tritrichomonas foetus*, a causative agent of a venereal disease of cattle, uses lactoferrin and transferrin as a source of iron. However, *T. vaginalis* utilizes lactoferrin-bound iron only. Therefore, *T. vaginalis* is colonized mainly at vaginal mucus where is lactoferrin-rich in condition [10].

Women with trichomoniasis may notice pruritic genitalium, burning sensation, or leucorrhea, and these symptoms often alter with the menstrual cycle. This phenomenon is presumably due to an elevated vaginal pH during menstruation that may promote *T. vaginalis* growth [11]. Iron was also presumed to contribute these symptom changes [2]. During menstruation, *T. vaginalis* may be exposed to a lot of iron in menstrual blood; therefore, changes of the menstrual cycle may affect the growth and pathogenicity of *T. vaginalis* [8]. The symptom presentation in trichomoniasis is also associated with not only the number of trophozoites but also the virulence of parasites. In a previous study, *T. vaginalis* in iron-rich conditions showed increased cytoadherence, cytotoxicity and subcutaneous ab-

scess formation in mice and increased PFOR activity. However, it was not clarified what molecules of *T. vaginalis* were involved in this mechanism [2,12].

Changes of protein expression of *T. vaginalis* by addition of iron to culture medium were reported [3]. When *T. vaginalis* was cultivated in iron-rich medium, over-expressed 120 kDa protein was observed, and cytoadherence to HeLa cells was increased. Over-expressed protein of *T. vaginalis* was identified as PFOR by MALDI-TOF MS. These results were consistent with this experiment.

In trichomonads infection, various factors such as adhesin, temperature, pH, cytoskeleton, and cysteine proteinase were involved in cytoadherence to mucous epithelial cells [13-15]. There are only few studies about the influence of iron on factors involving cytoadherence of *T. vaginalis*. Previous studies have shown that antibodies against *T. vaginalis* 120 kDa protein were involved in adhesion to HeLa cells [3]. In this study, reduction of adhesion of *T. vaginalis* to human vaginal epithelial cells (MS74) by anti-120 kDa Ab was shown. Antibody against 120 kDa protein of *T. vaginalis* also inhibited proliferation of trichomonads and subcutaneous abscess formation in mice. Taken together, iron in culture medium stimulates expression of 120 kDa PFOR in *T. vaginalis*, and this protein may be involved in the pathogenicity of *T. vaginalis*.

The menstrual cycle was known to closely interrelate with the survival and pathogenicity of *T. vaginalis*. Demes et al. [16] reported that the numbers of vaginal pathogens, including *T. vaginalis*, were decreased due to increased hemolytic complement in blood during menstruation. Increased blood in vagina during menstruation, however, may provide sufficient iron to *T. vaginalis*. The results of this experiment support the relationship of the pathogenicity of *T. vaginalis* with iron.

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## CONFLICT OF INTEREST

We have no conflict of interest related to this work.

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