The International Journal of Biochemistry & Cell Biology 40 (2008) 2442–2451

Contents lists available at ScienceDirect

The International Journal of Biochemistry & Cell Biology

journal homepage: www.elsevier.com/locate/biocel

Polyamine depletion down-regulates expression of the *Trichomonas vaginalis* cytotoxic CP65, a 65-kDa cysteine proteinase involved in cellular damage

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ARTICLE INFO

Article history:
Received 3 December 2007
Received in revised form 7 April 2008
Accepted 8 April 2008
Available online 24 May 2008

Keywords:
Trichomonas vaginalis
Cysteine proteinases
Cytotoxicity
Gene regulation by polyamines
Putrescine

ABSTRACT

Recently, we found that inhibition of putrescine synthesis by ornithine decarboxylase (ODC) significantly increased *Trichomonas vaginalis* adherence mediated by protein adhesins. Surprisingly and unexpectedly, trichomonal contact-dependent cytotoxicity was absent. Therefore, a role for polyamine depletion on regulation of *T. vaginalis* cytotoxicity mediated by the cysteine proteinase (CP) of 65-kDa, CP65, was investigated. We performed cytotoxicity and cell-binding assays followed by zymograms, as well as Western blot and indirect immunofluorescence assays using specific anti-CP65 antibodies to detect CP65. Trichomonads grown in the presence of the ODC inhibitor, 1-4-diamino-2-butanone (DAB) had lower levels of cytotoxicity that corresponded with diminished CP65 proteolytic activity when compared to untreated organisms handled identically. Likewise, semiquantitative and qRT-PCR as well as Western blot and immunofluorescence assays showed decreased amounts of *tvcp65* mRNA and CP65 protein in DAB-treated parasites. These effects were reversed by addition of exogenous putrescine. These data show a direct link between polyamine metabolism and expression of the cytotoxic CP65 proteinase involved in trichomonal host cellular damage.

Abbreviations: α-TUB, α-tubulin protein; *β-tub*, *β-tubulin* gene; B-TUB9, sense primer to amplify the *β-tubulin* gene; B-TUB2, antisense primer to amplify the *β-tubulin* gene; CPs, cysteine proteinases; CP65, the cytotoxic cysteine proteinase of 65-kDa with affinity to the surface of HeLa cells; CP39, cysteine proteinase of 39-kDa with affinity to the surface of HeLa cells; CP30, cysteine proteinase of 30-kDa with affinity to the surface of HeLa cells; CNCD, Centro Nacional de Clinica de Displasias; DAB, 1,4-diamino-2-butanone; DFMO, DL-α-difluoromethylornithine; DMEM, Dulbecco's Modified Eagle medium; HIV, human immunodeficience virus; IgG, immunoglobulin G; ODC, ornithine decarboxylase; NRS, preimmune normal rabbit serum; qRT-PCR, quantitative RT-PCR; S-65, antisense primer to amplify a fragment of the *tvcp65* gene; SSY65, sense primer to amplify a fragment of the *tvcp65* gene; STI, sexually transmitted infection; TCA, trichloroacetic acid; *tvcp65*, gene encoding the cytotoxic CP65 proteinase; TLR2, toll-like receptor 2; TYM, trypticase-yeast extract-maltose; TYM-serum, TYM medium supplemented with 10% heat-inactivated horse serum; VECs, vaginal epithelial cells.

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doi:10.1016/j.biocel.2008.04.023

1. Introduction

Trichomonas vaginalis is the human protozoan parasite responsible for trichomonosis, the most common sexually transmitted infection (STI). This STI caused by *T. vaginalis* is associated with adverse health consequences to women, which include infertility (El-Shazly et al., 2001), atypical pelvic inflammatory disease (Moodley et al., 2002), preterm delivery of low birth weight infants (Cotch et al., 1997), and cervical neoplasia (Viikki et al., 2000). Apart from urethritis in men, a relationship now has been established between serum antibodies to *T. vaginalis* and prostate cancer (Sutcliffe et al., 2006). This STI is more prevalent in disadvantaged communities (Sorvillo et al., 1998) and increases predisposition to human immunodeficience virus (HIV) seroconversion (Guenthner et al., 2005; Mason et al., 2005; Rughooputh and Greenwell, 2005). The high incidence and prevalence of this STI on the human population (Van Der Pol et al., 2005) make the study of *T. vaginalis* highly significant.

Successful host parasitism is achieved by parasite penetration of the mucus layer (Lehker and Sweeney, 1999) followed by adherence to vaginal epithelial cells (VECs) mediated by at least five protein adhesins (Arroyo et al., 1992; Moreno-Brito et al., 2005) and the proteolytic activity of at least two cysteine proteinases (CPs), CP30 and CP62, is needed (Arroyo and Alderete, 1989, 1995; Mendoza-López et al., 2000; Hernandez et al., 2004). Brief contact of *T. vaginalis* with VECs results in dramatic morphological changes from ellipsoid to amoeboid (Arroyo et al., 1993). Iron concentrations and cell contact upregulate adhesins expression in a coordinated fashion (Arroyo et al., 1993; García et al., 2003; Lehker et al., 1991). *T. vaginalis* cytoadherence modulates the expression of numerous parasite and host cell genes (Kucknoor et al., 2005a,b). Involvement of several CPs, some of which are directly implicated in contact-dependent cytolysis, contributes to *T. vaginalis* pathogenesis (Alvarez-Sánchez et al., 2000; Arroyo and Alderete, 1989, 1995; Hernández-Gutiérrez et al., 2003, 2004; Mendoza-López et al., 2000). The CPs of 65-kDa (CP65) (Alvarez-Sánchez et al., 2000, 2007; Solano-González et al., 2006) and 39-kDa (CP39) (Hernández-Gutiérrez et al., 2003, 2004) are involved in cytotoxicity. Both are surface-expressed and bind to the surface of human vaginal and cervical cells (Alvarez-Sánchez et al., 2000; Hernández-Gutiérrez et al., 2003, 2004). In particular, CP65 is active at pH and temperature of the infected vagina, degrades extracellular matrix proteins collagen IV and fibronectin, is immunogenic in patients with trichomonosis (Alvarez-Sánchez et al., 2000), and is down-regulated by iron, greatly reducing the trichomonal CP65-dependent cytotoxicity (Alvarez-Sánchez et al., 2007).

The polyamines putrescine, spermidine and spermine are abundant small cations found in all living species. These polycations are important multifunctional cellular components that are considered critical regulators of cell growth, division, differentiation and apoptosis. The intracellular concentration of polyamines is finely regulated by biosynthetic and metabolizing enzymes as well as by transport systems (Wallace et al., 2003).

The lead enzyme of polyamine biosynthesis of many cells is ornithine decarboxylase (ODC), which forms putrescine. *T. vaginalis* differs from other eukaryotes in several aspects of its polyamine metabolism. The putrescine formed from ornithine by ODC is not metabolized further but exported with the simultaneous uptake of spermine. Putrescine can be considered the end product of an energy-generating pathway (arginine dihydrolase pathway) and is found in large amounts in vaginal secretions of trichomonosis patients (Reis et al., 1999; Yarlett and Bacchi, 1988, 1994). *T. vaginalis* is unable to synthesize spermine; instead, spermine has to be obtained from the host through a putrescine/spermine antiporter system (Yarlett and Bacchi, 1994). Thus, putrescine is exchanged in a 2:1 molar ratio with the host-produced spermine, which is back-converted to spermidine by *T. vaginalis* (Yarlett and Bacchi, 1994; Yarlett et al., 2000). While inhibition of *Tritrichomonas foetus* ODC with 1,4-diamino-2-butanone (DAB) led to growth arrest, destruction of hydrogenosomes, and reduction of hydrogenosomal enzymes (Reis et al., 1999). For *T. vaginalis*, inhibition of ODC with DAB also resulted in growth arrest but there was no effect on the number and integrity of hydrogenosomes (García et al., 2005). Furthermore, no difference in the amount of adhesins (AP65, AP51, and AP33) between normal and DAB-treated organisms was observed, even though there was up to a 20-fold increase in the levels of cytoadherence (García et al., 2005). Remarkably and unexpectedly, the parasite contact-dependent cytotoxicity (Alderete and Pearlman, 1984; Krieger et al., 1985) was absent, a finding consistent with reduced trichomonal cytotoxicity due to DL-α-difluoromethylornithine (DFMO) treatment, another ODC inhibitor (Bremner et al., 1987).

The link between polyamine metabolism with *T. vaginalis* cytoadherence and cytotoxicity (García et al., 2005), together with the involvement of thiol proteinases in contact-dependent cytotoxicity (Alvarez-Sánchez et al., 2000, 2007; Arroyo and Alderete, 1989, 1995; Hernández-Gutiérrez et al., 2003, 2004; Solano-González et al., 2006), led us to hypothesize that polyamine depletion was linked to trichomonal cytotoxicity through the regulation of CP65 gene (*tvcp65*) expression, affecting the synthesis and proteolytic activity of CP65. Therefore, we tested whether polyamine depletion regulates CP65 expression and the CP65-dependent cellular damage inflicted by *T. vaginalis* to the host cells.

In this report we show that DAB treatment of *T. vaginalis* down-regulates CP65 expression concomitant with the reduction of the CP65-dependent host cytotoxicity. To our knowledge, this is the first report that shows a direct link between trichomonal polyamine depletion and the down-regulation of *tvcp65* expression, the gene encoding the cytotoxic CP65 proteinase.

2. Materials and methods

2.1. Culture and growth of T. vaginalis

The fresh clinical *T. vaginalis* isolate CNCD 147 was used in this study (Alvarez-Sánchez et al., 2000, 2007; Solano-González et al., 2006). Parasites were grown up to 3 weeks by daily passage in trypticase yeast extract maltose (TYM)