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Mini review

The complex fibronectin–*Trichomonas vaginalis* interactions and Trichomonosis

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Abstract

Trichomonosis is the vaginitis caused by *Trichomonas vaginalis*. This sexually transmitted agent achieves successful host parasitism through various means including: (1) acquisition of nutrients through specific receptors; (2) recognition and binding to mucin followed by cytoadherence mediated by adhesins that resemble metabolic enzymes; (3) evasion of immune responses through (i) masking of organisms by host proteins, (ii) shedding of trichomonad proteins into the secretions and (iii) secretions of cysteine proteinases that degrade all immunoglobulin subclasses and complement; (4) alternating surface expression of at least two antigen repertoires; and (5) alternate and coordinate expression of virulence genes in response to host environmental factors. The fact that the parasite survives long term in the varying and adverse environment of the vagina attests to the highly evolved nature of this protist. An understanding of the non-self-limiting nature of this infection may come from recent findings illustrating the complexity of *Trichomonas vaginalis*–fibronectin (FN) interactions. The parasite readily attaches to surfaces with immobilized FN and binds to FN in a highly specific receptor-mediated fashion. The amount and affinity of bound FN by live organisms is influenced by concentrations in medium of both iron and calcium. De novo protein synthesis is required for optimal FN acquisition in the presence of calcium. Furthermore, the parasites bind with differing affinities to the N-terminal domain (NTD), the cell-binding domain (CBD) and the gelatin-binding domain (GBD) of FN. Iron modulates binding of NTD similar to that of FN. This minireview summarizes recent findings on the *T. vaginalis*–FN associations.

Keywords: Calcium; Parasitism; Extracellular matrix; Fibronectin; Iron; *Trichomonas vaginalis*

Abbreviations: CBD, cell-binding domain; ECM, extracellular matrix; FN, fibronectin; GBD, gelatin-binding domain; LM, laminin; NTD, N-terminal domain; VEC, vaginal epithelial cell.

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less of the iron on growth medium, further indicated an advantage to low-iron organisms incapable of cytoadherence. In this scenario, parasites poorly adhering to VECs still had potential for successfully colonizing the host.

These observations were a natural first step in demonstrating the ability of live *T. vaginalis* organisms to interact with FN. However, during these experiments, we hypothesized that associations of parasites with ECM components would promote growth of trichomonads. This was tested taking into account the density-dependent growth properties of trichomonads in 96-well micro-dilution plates. Seeding at <100 organisms per well results in eventual death of the parasite. The inability of parasites to grow reproducibly during single-cell cloning or at densities <100 per well could not be reversed by the addition of: conditioned medium; reducing reagents; and growth factors. In contrast, reproducible single cell growth was obtained in microtiter wells containing immobilized FN. This apparent growth stimulation was completely abolished by pretreatment of cover slips with anti-FN antibody alone, affirming the role of FN in growth initiation. Whether the trichomonad-FN interaction involves signal transduction pathways regulating cell cycle progression requires experimental verification. Nonetheless, these findings are provocative and may have significance in terms of in vivo growth stimulatory effectors.

3. Specific FN acquisition by live trichomonads is regulated by iron and calcium

In vitro experiments were then performed to characterize the acquisition of FN by live and fixed trichomonads [8]. Immunofluorescence (Fig. 1a) and immuno-gold labeling (Fig. 1b) readily demonstrated FN on the parasite surface. Acquisition of FN was specific and saturable. Once bound onto the trichomonal surface, FN could be released through the action of the proteinases secreted by the parasite. Therefore, it must not be overlooked that the numerous membrane-associated [14,15] and/or secreted [4,16] trichomonad proteinases may play a role in modulating the interaction between parasite and either cellular or ECM and BM FN. For this reason, experiments were performed comparing acquisition of FN by live vs. fixed trichomonads. Saturation binding was achieved for both, although, not surprisingly, lower amounts of FN bound to live organisms. Importantly, competition by unlabeled FN, but not another unrelated glycoprotein, of iodinated FN was obtained, showing the specific nature of FN acquisition. Additional competition experiments were performed using the cell-binding domain (CBD) of FN and the integrin-binding peptide RGD contained within the CBD. The RGD produced no noticeable competition while the CBD decreased the amount of iodinated FN by 50%, indicating that non-RGD sequences and possibly sites outside the CBD were being recognized.

Interestingly, high-iron-grown trichomonads acquired with higher affinity lower numbers of FN molecules than low-iron-grown counterpart organisms. Table 1 summarizes data from a representative experiment and shows that low-iron parasites bound 4.8×10^5 FN molecules, more than twice that bound by high-iron parasites. The binding affinity for high iron was 20-fold greater than seen for low iron. In the presence of calcium and as reported recently [6], the binding affinities for high- and low-iron-grown trichomonads were as shown in Table 1. However, the overall number of FN molecules acquired by live organisms were increased fourfold for high-iron-grown trichomonads and over twofold for low-iron-grown parasites, thus approaching equal numbers of FN molecules (2×10^6) bound regardless of the iron status.

Furthermore, the mechanism by which calcium exerted its effect was rapid and required de novo protein synthesis. The identity of the newly synthesized protein or proteins required for FN acquisition remains unknown. This may be the first time that calcium has been shown to regulate a property for *T. vaginalis*. A phosphatase, but not protein kinase C, was shown to be involved in optimal FN binding, suggesting phosphorylation and signaling pathways may play a role in this aspect of host parasitism. These newly synthesized proteins and the pathways induced by calcium, once dissected, may provide future targets for drug intervention. Lastly, it is noteworthy that the iron

FN molecule [8]. Saturation binding of *T. vaginalis* to each of the three domains of FN was achieved. In this type of experiment, 5×10^5 trichomonads were incubated in a 100 μl volume of buffer containing inhibitors of cysteine proteinases [8] and increasing amounts of each iodinated domain. Data from a representative experiment indicated that live trichomonads bound $\sim1.5\times10^6$ molecules of NTD and GBD each compared to $\sim2.5\times10^5$ for CBD and FN. Interestingly, the amount of NTD bound was dependent on iron. As seen for the intact FN molecule, the NTD was acquired in lower amounts and with higher affinity among high-iron-grown compared to low-iron-grown trichomonads. The binding affinities (K_D) defined as the amount (nM) of iodinated FN or domains required to reach half saturation binding were calculated to be: 0.02 for FN; 0.50 for CBD; 2.00 for NTD; and 1.60 for GBD, showing that the K_Ds for the individual domains were lower than that for FN. Importantly, only the unlabeled NTD, GBD and CBD competed for binding with the homologous domain, showing specificity in the interaction. Furthermore, in competition experiments involving the domains and intact FN, only CBD effectively competed for FN acquisition, suggesting that it is the primary domain recognized by trichomonads.

Finally, as shown in Fig. 2, trichomonads readily attached to FN and each of the domains immobilized on cover slips. After 30 min incubation on cover slips, the parasites were fixed to the cover slips where, after treatment with buffer containing bovine serum albumin, bound organisms were visualized by fluorescence after treatment with rabbit antiserum to *T. vaginalis*. Prebleed control rabbit serum was unreactive and gave no fluorescence. Interestingly, in a property similar to that observed upon cytoadherence of VECs with live *T. vaginalis* organisms [18], trichomonads attached to FN and the NTD underwent morphologic transformation from ellipsoid to amoeboid morphology with numerous pseudopodia and membrane extensions (Fig. 2b). Fewer membrane extensions were evident for CBD and GBD, possibly illustrating the specificity in signaling.

5. Trichomonads have FN-like iron-regulated proteins

As the associations between *T. vaginalis* organisms and FN is complex, and given the evidence for specific receptor-ligand type interactions [8], an attempt was made to identify genes of *T. vaginalis* that encode for FN-binding proteins. The idea was to use the ability to bind FN as a capture assay for identification of cDNA clones in an expression library. One such cDNA clone called C1 was obtained based on FN binding [19]. This cDNA represented an incomplete gene, and not surprisingly, the recombinant fusion protein bound FN by ligand-blotting, an assay in which anti-FN antibody detected FN incubated with recombinant protein immobilized onto nitrocellulose after SDS-PAGE. Surprisingly and unfortunately, further cloning of cDNA C1 showed the FN-binding activity to be artificial, resulting from fusion at the EcoRI site of the LacZ gene carried on the expression vector pcDNAII (InVitrogen). Nonetheless, the full-length 378-bp gene encoding a 14.8 kDa protein of 125 amino acids was obtained and characterized further. The amino acid sequences revealed homology with the type III-14 repeat of the heparin-binding domain at the carboxyl terminal end of FN. This fibronectin-like protein gene, flp1, was single copy in all the *T. vaginalis* isolates examined, and flp1 mRNA levels were elevated when organisms were grown in low-iron-medium. During the course of recovering the full-length gene by PCR using genomic DNA, another low-iron-regulated gene, flp2, with 70 and 67.5% identity to flp1 at the nucleotide and amino acid levels, respectively, was recovered. Both flp1 and flp2 had consensus Inr promoter-like elements immediately adjacent to the start codon. The flp2 genomic sequence also contained an additional Inr element followed by an ATG 24-bp within the gene. Unlike flp2, flp1 had AU-rich destabilizing elements in the 3′-untranslated region (UTR), and the occurrence of similar elements is common to other trichomonad transcripts [20].

The discovery of these two genes was noteworthy for several reasons. They were the first low-iron-regulated trichomonad genes to be reported [19], and as such have become important in studies

tity of any surface FN-binding proteins remains unknown. Also unknown is whether the interaction between the trichomonal surface and FN is through single or multiple domains on the intact FN molecule. The data indicate that distinct sites exist for recognition and binding to three FN domains, and the data show that FN and domains bind with relatively high affinity. It is noteworthy that the binding affinities for the individual domains were lower than that for FN. It is conceivable that high affinity binding by the FN molecule is the cumulative result of recognition of all domains concurrently. It is well recognized that many pathogens interact with multiple FN domains. Gram-positive bacteria, such as *S. aureus*, recognize both NTD and the high affinity heparin-binding domain located at the C-terminal end of FN [21]. Similarly, protein F1 of *S. pyogenes* possesses two FN-binding domains, one recognizing NTD and the other a 70 kDa region encompassing both NTD and GBD [22]. Other organisms, such as *T. cruzi*, bind to the CBD via recognition of the integrin-binding RGD peptide [23]. Equally noteworthy is the fact that other organisms modulate their ability to bind FN by iron. For example, *Candida albicans* preferentially binds GBD when grown in complex medium, whereas when cultured in a defined medium supplemented with hemoglobin as a source of iron binds CBD [24].

Finally, it would be surprising if *T. vaginalis* would not possess multiple mechanisms for colonization of the vaginal tract. Without a doubt, the interactions with mucin, VECs and ECM molecules confer upon this parasite the amazing capacity to persist in a non-self-limiting infection. When viewed in a holistic fashion, possessing multiple mechanisms for host parasitism through interactions with different substrates, and where such interactions are known to be or may be under the control of environmental factors, such as iron and calcium, *T. vaginalis* is highly evolved and sophisticated ancient protist.

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