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Induction of mitotic S-phase of host and neighboring cells by *Toxoplasma gondii* enhances parasite invasion

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Abstract

The intracellular parasite *Toxoplasma gondii* extensively modifies its host cell so as to efficiently grow and divide. Among these cellular changes, *T. gondii* alters the cell cycle of host cells it has invaded. We found that *T. gondii* affects the cell cycle of not only the cells it directly invades, but neighboring cells as well. Both direct invasion by *T. gondii* and exposure to filtered medium from cultures of *T. gondii*-infected cells (conditioned medium) caused normally quiescent fibroblasts to enter S-phase. *T. gondii* has been shown to attach to and invade S-phase host cells more readily, and we found that conditioned medium increased the rate of invasion of *T. gondii* into new host cells. Thus it appears that *T. gondii* directly releases, or induces parasitized host cells to release, a factor that induces neighboring cells to enter S-phase, allowing more rapid invasion by extracellular *T. gondii* and providing a possible selective advantage for the parasite.

Toxoplasma gondii is an obligate intracellular parasite in the phylum Apicomplexa. Upon entering a host cell, *T. gondii* causes changes in transcription and protein expression patterns of a wide range of host genes [1,2]. These include proteins with known functions in such processes as metabolism, transcriptional regulation, cell signaling, inflammation, apoptosis, and the cell cycle. Indeed, several recent studies have reported that *T. gondii* invasion can influence the host cell cycle. Brunet et al. [3] determined cell cycle phase of *T. gondii*-invaded human dermal fibroblasts and human trophoblasts by measuring DNA content using flow cytometry of propidium iodide (PI) stained cells. They found that invasion by *T. gondii* caused rapidly dividing cells to arrest at the G2/M boundary, while quiescent fibroblast monolayers were induced to transition from G0/G1 to G2/M, where they also arrested. Molestina et al. [4] also investigated cell cycle phase of *T. gondii*-invaded cells using flow cytometry to determine DNA content of PI stained cells. This study found that *T. gondii* invasion induced human foreskin fibroblast monolayers to transition from G0/G1 to S-phase. However, they did not find that cells accumulated at the G2/M border, as did Brunet and colleagues, but rather remained in S-phase.

We have also observed that *T. gondii* invasion induced normally quiescent cells to enter S-phase. The S-phase marker bromodeoxyuridine (BrdU) is rarely incorporated into cells of a confluent monolayer of human foreskin fibroblasts (HFFs), as these cells experience contact inhibition and are in G0-phase. However, we observed that BrdU was incorporated into a significantly larger proportion of cells when the tissue culture was infected by RH strain *Toxoplasma gondii*. In control uninfected cells allowed to incorporate 10μM BrdU for 1hr and

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then stained with an anti-BrdU antibody, only 0.2% of cells were BrdU positive, compared with 8.6% in *T. gondii* infected tissue cultures (Fig. 1A–B). This result indicates that as a consequence of the infection cells transition to S-phase in response to *T. gondii* despite remaining in contact with other fibroblasts in the monolayer (Fig. 1A–B). Because we used a relatively low MOI in these experiments (approximately 0.8), we were able to view both parasitized and unparasitized HFFs in *T. gondii*-exposed monolayers. Interestingly, we noted that not only cells with visible internal parasites but also neighboring cells that had no sign of being invaded showed the S-phase specific marker (Fig. 1A). When we counted only the HFFs that had been invaded by *T. gondii*, 24% were positive for BrdU (Fig. 1B). However, of the remaining HFFs in these wells, which were not directly invaded by *T. gondii*, 6.4% were BrdU⁺ (Fig. 1B). This was still significantly greater than the percentage of BrdU⁺ HFFs in control wells. In addition the BrdU⁺ HFFs that had not been invaded were not necessarily in direct contact with a parasitized HFF. This suggested that a soluble factor released by the parasites or by the infected cells could be responsible for induction of fibroblasts into S-phase.

To explore this possibility, we isolated parasite-conditioned medium (cell culture medium that had been removed from HFFs 24hrs post-invasion and passed through a 0.22µm filter to eliminate the parasites), and compared its ability to induce S-phase in a confluent monolayer with that of control medium (filtered medium from unparasitized HFFs). In brief, confluent HFFs were exposed to either parasite-conditioned or control medium for 24hrs, then exposed to BrdU for 1 hour and stained with anti-BrdU antibodies. In this manner we observed that 13% of the cells exposed to parasite-conditioned medium (CM) were BrdU positive, compared to only 0.2% for the control cells (Fig 1A and C). Thus, a released diffusible factor from either the infected cells or the parasites themselves is responsible for the change in cell cycle status. To test whether the accumulation of such a factor was specific to *T. gondii* infection, or a general non-specific consequence of stressed or damaged HFFs, we examined the ability of conditioned medium from heat-shocked, UV irradiated, or virally infected HFFs to induce DNA synthesis. Conditioned media from these treatments did not produce a significant effect on BrdU incorporation by confluent HFFs (Fig. 1C), indicating that the effect seen is specific to infection by *T. gondii*. Brunet et al. [3] reported that unlike parasitism, exposure to CM did not cause arrest of rapidly dividing trophoblasts at G2. Nonetheless, they did not examine whether CM induced quiescent cells in monolayers to transition to G2, as we have done. Here we find that conditioned medium alone can induce transition to S-phase in quiescent confluent cells.

In order to begin identification of the principal agent(s) in CM responsible for S-phase induction in HFFs, we compared BrdU incorporation caused by CM with boiled CM, and CM that had been separated by 10kDa nominal molecular weight centrifugal filters. Incubating CM at 100°C for 5 min completely blocked its ability to induce BrdU incorporation in confluent HFF monolayers (Fig. 1D). When CM was separated using a 10kDa molecular weight filter, the activity was lost in the <10kDa fraction, but retained and concentrated in the >10kDa fraction (Fig. 1D). Thus, the S-phase inducing factor(s) in conditioned medium is heat-labile and greater than 10kDa in size.

It is possible that the factor(s) present in the conditioned media that induced HFFs to enter S-phase was of either parasite or host cell origin. Previously Blader et al [1] found that extracellular parasites that were physically separated from potential host cells by a membrane that was permeable to *T. gondii*-secreted proteins and other molecules, but not the parasites themselves, induced expression of a substantial and specific set of genes in these potential host cells. Thus parasite-secreted factors are capable of altering host cell gene expression without the need for the parasite to physically invade or even contact the cell. To investigate whether such parasite-secreted factors contributed to the observed induction of S-phase in HFFs, we used the technique of Blader et al. [1] and physically separated parasites from HFFs using a

permeable support with a 0.4 μ M pore-size membrane (Corning Transwell). Parasites were placed in the chamber above the membrane, allowing PSFs to diffuse to the HFF monolayer in the lower chamber but preventing the parasites from either invading or directly contacting the HFFs. HFFs exposed to the PSFs for 24hrs though a membrane did not differ in BrdU incorporation from controls (Fig 1C). Although this does not rule out a factor of parasite origin being responsible for S-phase induction, it does suggest that the release of the factor, whether from the parasite or host, requires either direct contact between the parasite and the host cell or invasion.

T. gondii can successfully invade and develop in virtually any mammalian or avian nucleated cell [5,6]. However, the mechanism *T. gondii* uses to recognize and attach to a new host cell is only now being elucidated. It appears that sulfated proteoglycans may serve as receptors on host cells [7,8], although other reports suggest they may not be important as receptors but instead play a role in post-invasion replication [9]. Whatever host cell-surface molecules are important in recognition, it appears that the cell cycle influences their expression. *T. gondii* more readily attaches to and invades host cells in S-phase than in other cell cycle phases [10, 11], and antibodies generated against S-phase surface molecules are particularly effective in blocking invasion [11]. Given these observations we would expect CM, which promotes entry of fibroblasts into S-phase, to promote invasion of host cells by tachyzoites. To test this, we replaced the normal medium from HFF monolayers with CM and allowed the cells to incubate for 24hrs. We then switched the medium to normal medium and added parasites for either 10min, 1hr or 6hrs, and compared their success in invasion to that in untreated host cells. Since at the time points tested invaded and extracellular parasites are indistinguishable, we identified those parasites inside host cells by using a double staining method in which parasites were differentially labeled with antibodies for a surface antigen (SAG-1, gift from J. Boothroyd) before permeabilization of HFFs (which would only stain extracellular parasites) and after permeabilization (which would stain both intra- and extracellular parasites) [12]. Utilizing this method we determined the number of intracellular parasites in 20 randomly chosen fields of view (400x magnification) for CM-exposed and untreated control HFFs, and expressed our results as the ratio of the number of intracellular parasites in CM-exposed samples over the number of intracellular parasites in control samples (Fig. 2A). This ratio was highest (indicating more successful invasion of HFFs pre-exposed to CM) for the 10min time point, with a ratio of 2.44. By 1hr this ratio had decreased to 1.82, and by 6hrs it was 0.99, indicating there was no longer a difference in invasion of the CM-exposed and control HFFs. Thus CM aids in the rate of invasion of HFFs by *T. gondii*, but given longer time-periods parasites can invade at similar numbers whether or not HFFs were exposed to CM.

This last result shows that medium from infected tissues can result in a greater efficiency of invasion. Given that we have shown that conditioned medium induces cells to move into S-phase and that it has been previously reported that *T. gondii* invades more efficiently into S-phase cells [10,11], we propose that the effect of the conditioned medium on invasion is due to the increased number of cells entering S-phase. Grimwood et al [11] showed that the greater efficiency of *T. gondii* invasion of S-phase cells was apparently a result of more efficient attachment of parasites to a receptor that was upregulated during mid S-phase. Thus, we expected that after pre-treatment with conditioned media in our invasion assays, parasites would be more likely associated with BrdU⁺ cells (i.e. those in S-phase) than BrdU⁻ cells. We therefore investigated the rates of parasite attachment and/or invasion to individual HFFs that had been exposed to CM and BrdU, so we could directly correlate attachment/invasion rate with induction into S-phase. HFFs were incubated in CM for 24hrs and then incorporated 10 μ M BrdU for 1hr. 2×10^6 RH strain *T. gondii* were added per well of a 24-well tissue culture plate and incubated with the HFFs at 37°C for 10min. Cells were washed 5 times in PBS and then fixed and stained using anti-BrdU antibodies, and the parasites were visualized using an anti-SAG1 antibody. As can be seen in Fig. 2B, visual inspection of the stained cells shows

that parasites are more likely to be associated (either attached or invaded) with those cells that stain positive for BrdU. In order to quantify this effect, we determined the mean number of parasites (MNP) per BrdU⁺ cell and the MNP per BrdU⁻ cell for at least 100 total cells, and expressed the data as the ratio of MNP per BrdU⁺ cell over MNP per BrdU⁻ cell. This ratio was 4.86 ± 0.88 (mean $\pm$ 1 standard deviation for three independent experiments), indicating that BrdU⁺ cells had nearly 5 times as many associated parasites as BrdU⁻ cells in the same tissue culture. Thus the effect of conditioned medium on the cell cycle results in increased invasion efficiency.

Our finding that cells parasitized by *T. gondii* release a factor capable of inducing uninfected cells to enter S-phase links reports of cell cycle arrest in *T. gondii*-infected cells [3,4] with reports that *T. gondii* more readily invades cells in S-phase [10,11]. There may be multiple advantages for *T. gondii* in arresting host cell cycle. For instance, Brunet et al. [3] found that preventing S-phase induction of host cells, by artificially arresting them at G1 by means of RNAi inhibition of the cellular growth regulator UHRF1, interfered with *T. gondii* development. Here we show another advantage for the parasite, in that *T. gondii* infection can result in S-phase induction of neighboring, uninfected cells, and that this allows more rapid adhesion and invasion of new host cells as the parasites egress and move to invade a new host cell. While the effect of conditioned-medium treatment is only a 2.44 fold increase in invasion efficiency, a two-tailed T-test finds this to be a significant difference at a 95% confidence level ($P=0.012$). The relatively small effect on invasion might be due to the fact that we are analyzing invasion efficiency in a mixed population of host cells – although conditioned medium increases the proportion of cells entering S-phase, it does not cause all cells to enter S-phase. Indeed, when we subsequently examined the efficiency of attachment of parasites to individual cells in a conditioned medium treated-well, we found 4.86 times as many parasites attached to S-phase cells as compared to non-S-phase cells. In previous reports of preferential attachment of *T. gondii* to S-phase cells, Dvorak and Crane [10] found about a 4-fold increase in association of *T. gondii* with HeLa cells in synchronous S-phase as compared with synchronous G1-phase. Grimwood et al. [11] found about a 3-fold increase in association of *T. gondii* with MDBK cells in synchronous S-phase as compared with synchronous G1-phase. When compared to asynchronous MDBK cultures, synchronous S-phase cells showed only about a 1.3-fold increase in association of *T. gondii* [11]. Thus our results seem to correspond both in nature and degree with previously published reports of preferential association of *T. gondii* with host cells in S-phase.

The observed increase in parasite efficiency of invasion of HFFs that had been exposed to conditioned medium is relatively brief, and by 1hr there are no longer significant differences between CM-exposed and control HFFs. This may be because although HFFs not exposed to CM would express fewer receptor molecules for invasion, by 6hrs most parasites that remained viable would have been able to locate such a receptor. Alternatively, by 6hrs invaded cells or parasites may be producing enough factors to cause S-phase induction in neighboring cells, rendering them better invasion targets. In any case, even a brief temporal advantage in invading host cells could be of significant selective advantage to the parasite, which would spend less time in the extracellular environment where parasites can be readily attacked by antibodies and other components of the host's humoral immune response [13].

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Abbreviations

HFF	human foreskin fibroblast
CM	conditioned medium
BrdU	bromodeoxyuridine
PSFs	parasite secreted factors
MNP	mean number of parasites

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Figure 1.

Invasion by *T. gondii* and exposure to parasite-conditioned medium (CM) promote BrdU incorporation by confluent HFFs. (A) Cells were exposed to filtered cell culture medium, RH strain *T. gondii*, or filtered CM (from cells infected for 24hrs) for 24hrs, then incubated in 10 μ M BrdU for 1hr. BrdU positive nuclei were visualized by staining with a BrdU antibody and a red fluorescent-tagged secondary antibody. Arrows indicate *T. gondii* vacuoles. (B) Percentages of BrdU positive cells for control HFFs, all HFFs in tissue culture wells to which RH strain *T. gondii* had been added (parasitized – all HFFs), HFFs in these same wells that had been directly invaded by *T. gondii* (parasitized – invaded HFFs), and HFFs in these same wells that had not been invaded by *T. gondii* (parasitized – non-invaded). Data are means from three independent experiments. Each error bar equals 1 standard deviation. * indicates BrdU

incorporation significantly different from control ($P < 0.05$). (C) Percentages of BrdU positive cells for control HFFs, HFFs exposed to CM from *T. gondii* invaded cells, CM from HFFs infected with polio virus (MOI 1) for 24hrs and heat inactivated at 52°C for 2hrs, CM from HFFs that had been heat shocked at 42°C for 2hrs then incubated at 37°C for 24hrs, CM from HFFs that had been exposed to 254nm ultraviolet light from a 30W germicidal mercury-vapor lamp for 30min at 25°C and then incubated at 37°C for 24hrs, and parasite secreted factors (PSF - see text for details). Each of the CM were added to HFFs for 24hrs and then incubated for 1hr in 10μM BrdU. Data are means from three independent experiments. Each error bar equals 1 standard deviation. * indicates BrdU incorporation significantly different from control ($P < 0.05$). (D) S-phase promoting activity of CM is principally due to a heat-labile molecule of molecular weight >10kDa. HFFs were exposed to CM, boiled CM, or the filtrates and retentates of CM passed through a 10kDa molecular weight cutoff filter. HFFs were then incubated for 1hr in 10μM BrdU. Data are the means of percentage of cells positive for BrdU incorporation from three independent experiments. Each error bar equals 1 standard deviation. * indicates significant difference from untreated CM ($P < 0.05$).

Figure 2.

Invasion and adherence to HFFs are enhanced by conditioned medium. (A) Equal numbers of RH strain *T. gondii* were allowed to invade HFFs (which had been exposed to control or conditioned media) for the indicated time period. The data were expressed as the total number of parasites that had invaded CM-exposed cells divided by the total number that had invaded control cells, with a ratio of 1 thus indicating no difference in invasion efficiency between conditioned and control media. Data are means from three independent experiments. Each error bar equals 1 standard deviation. * indicates tachyzoite invasion differs significantly different from control ($P < 0.05$). (B) Micrographs of the same field of view showing the preferential adherence of *T. gondii* tachyzoites to BrdU positive HFFs after 10min invasion time. Tachyzoites were stained with anti-SAG1 antibodies. In the phase contrast view, black arrows highlight HFFs.