Susceptibility of Retinal Vascular Endothelium to Infection with Toxoplasma gondii Tachyzoites

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PURPOSE. Retinochoroidal infection with the protozoan parasite Toxoplasma gondii is the most common cause of posterior uveitis worldwide. Tachyzoites spread throughout the body through the blood stream and lymphatics, but preferentially encyst in the eye and other parts of the central nervous system (CNS). It is unknown whether T. gondii penetrates the CNS selectively or whether these sites of immune privilege have limited capacity to eradicate the parasite.

METHODS. Human vascular endothelial cell lines, including retinal (three lines from three different donors), aortic, umbilical vein, and dermal microvascular endothelium, as well as human foreskin fibroblasts, were grown to confluence in 24-well plates. Cells were inoculated with RH-strain T. gondii tachyzoites in the presence of [3H]-uracil. Trichloroacetic acid-insoluble radioactivity was measured as an index of T. gondii proliferation, because tachyzoites, but not human cells, incorporate uracil directly through pyrimidine salvage.

RESULTS. Tachyzoites showed higher [3H]-uracil incorporation after incubation with retinal vascular endothelial cells in comparison with aortic (55% more), umbilical vein (33% more) and dermal (34% more) endothelial cells. In eight separate assays, significantly greater radioactivity was measured for tachyzoites cultured with retinal versus other cell subtypes (P < 0.05), except for one assay in which differences reached only borderline significance (P ≤ 0.07). In contrast, experiments comparing different retinal endothelial lines revealed no difference between any pair. Growth of the tachyzoites was approximately 2.8-fold higher in retinal endothelium than in foreskin fibroblasts, the cell subtype often used to investigate processes of T. gondii infection.

CONCLUSIONS. Enhanced susceptibility of retinal vascular endothelium to infection by T. gondii tachyzoites may explain, at least in part, preferential localization of T. gondii to the retina. Susceptibility may relate to preferential binding of tachyzoites to the retinal vascular endothelial surface, relative ease of penetration into the cell, rate of replication within the cell and/or cell response to infection. (Invest Ophthalmol Vis Sci. 2004;45:1157–1161) DOI:10.1167/iovs.03-1105

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Retinal infection with the protozoan parasite Toxoplasma gondii is one of the most common forms of posterior uveitis diagnosed in both Western societies and developing nations.1–3 T. gondii is an obligate intracellular protozoan for which humans may act as secondary hosts, acquiring the infection as a result of passage of tachyzoites across the placenta or through the ingestion of oocysts in fecally contaminated food or tissue cysts in undercooked meat.4–5 Tachyzoites, the actively dividing forms of the organism, disseminate throughout the body through the blood stream and lymphatics and invade both phagocytic and nonphagocytic cells. Multiplication of the tachyozoite within a cell leads to cell lysis, and daughter tachyzoites subsequently infect other cells or, finally, convert to tissue cysts or bradyzoites. Although tissues throughout the body may be infected, in the human, cysts are found to predominate in the eye and other parts of the central nervous system (CNS). It is not known whether T. gondii penetrates these areas relatively easily or whether these immunologically privileged sites do not eradicate the parasite.5–6 Tropism of different microorganisms for particular cell types and/or specific tissue sites is a long-recognized biological phenomenon, although underlying mechanisms may be poorly understood. Well-known examples include the special tropism of the hepatitis C virus for hepatocytes,6–7 preferential adherence of bacterium, Helicobacter pylori, to gastric mucosa8; and the specificity of the protozoan, Plasmodium vivax, to infect red blood cells.9 Recently, there has been interest in the heterogeneity of vascular endothelium, not only between arterial and venous types,10 but also between same type vessels located within different organs or within different tissues in the same organ.10,11 Research in the field of toxoplasmic retinochoroiditis has focused largely on the ocular microenvironment, as an explanation for localization of infection to the posterior eye.12–14 Because T. gondii tachyzoites must cross the retinal vascular endothelium irrespective of the mode and timing of infection, we hypothesized that preferential susceptibility of the retinal endothelium to infection contributes to the observed localization of the disease. Using a simple replication assay, exploiting the fact that tachyzoites, but not human cells, are capable of incorporating uracil directly through pyrimidine salvage,15 we investigated the susceptibility of human retinal vascular endothelium to tachyzoite infection in comparison to other vascular endothelial cell subtypes.

MATERIALS AND METHODS

Vascular Endothelial Cell Cultures

Human extraocular vascular endothelial cell cultures, isolated from aorta (17-year-old male donor), umbilical vein (newborn male donor) and dermal microvascularity (30-year-old female donor), were purchased from Cascade Biologics (Portland, OR). Three human retinal microvascular endothelial cell lines, isolated from three different donors (donor 1, 17-year-old female; donor 2, 18-year-old male, and donor 3, 14-year-old male) were obtained from the Applied Cell Biology Institute (Kirkland, WA). All endothelial cells were cultured in MCDB-131 medium (product number M-8537; Sigma-Aldrich, St. Louis, MO),
supplemented with 10 mM sodium bicarbonate, 10% fetal bovine serum (FBS) and endothelial growth factors and antibiotics (EGM-MV2 BulletKit, with omission of hydrocortisone and FBS; Clonetics-Cam- brex Inc., East Rutherford, NJ), at 37°C and at 5% CO₂. Human foreskin fibroblasts, also purchased from Cascade Biologies, were grown in DMEM (product number 12100; Invitrogen-Gibco Inc., Carlsbad, CA), supplemented with 40 mM sodium bicarbonate, 10% heat-inactivated FBS, 50 U/mL penicillin, and 50 μg/mL streptomycin (product number 15070; Invitrogen-Gibco Inc.), under identical conditions. Cell cultures were at passages 5 to 8 when used in the [3H]-uracil-incorporation tachyzoite replication assay.

T. gondii Tachyzoite Cultures

RH strain T. gondii tachyzoites were maintained by serial passage in T25 flasks containing confluent monolayers of human foreskin fibroblasts at 37°C and at 5% CO₂. Before fibroblasts were infected with tachyzoites, the percentage of FBS in the DMEM-based medium described earlier was reduced to 1%.

[3H]-Uracil Incorporation Assay

In three separate sets of experiments, each performed at least four times, T. gondii proliferation was compared in (1) human aortic endothelial cells, umbilical vein endothelial cells, and retinal endothelial cells (donor 1); (2) dermal endothelial cells and retinal endothelial cells (donor 1); and (3) retinal endothelial cells from all three donors. In all assays, human foreskin fibroblasts were included as a positive control for the infection. Vascular endothelial cells and human foreskin fibroblasts were grown to confluence in 24-well plates. Once cells were confluent, the medium was switched to MCDB-131, supplemented with 5% FBS and endothelial growth factors, but without antimicrobial agents. This medium ensured endothelial cell viability for the purposes of the assay, while at the same time facilitating T. gondii replication. The [3H]-uracil incorporation assay was performed according to a method adapted from Roos et al.¹⁵ In brief, cells were incubated with 2 × 10⁵ organisms in 1 mL of medium for 2 hours at 37°C. An aliquot of 5 μCi of [3H]-uracil (Moravek Chemicals, Brea, CA) was added to each well, and subsequently the plates were incubated for 18 hours at 37°C. Disruption of the cell monolayer during the incubation, although not quantified, was expected because tachyzoite proliferation within a cell ultimately leads to lysis of that cell. One milliliter of chilled 0.6 N trichloroacetic acid was added to each well, and the plates were incubated on ice for 1 hour. After removal of the trichloroacetic acid, the plates were washed overnight with water. The next day, 0.5 mL of 0.1 M sodium hydroxide was added to each well and the plates were incubated for 1 hour at 37°C. Radioactivity of samples, diluted 1:25 in acidified scintillation fluid (Ecolume; ICN Biomedicals Inc., Irvine, CA) was determined in a liquid scintillation counter. In each experiment, the assay was performed in triplicate, and the same number of negative controls, incubated in media without T. gondii, was always included.

Statistical Analysis

For each experiment, the levels of [3H]-uracil incorporation by tachyzoites growing in different cell types were compared by unpaired Student’s t test (two-tailed). P ≤ 0.05 or less was considered statistically significant.

RESULTS

In a series of four independent experiments comparing tachyzoite proliferation in retinal microvascular, aortic, and umbilical vein endothelial cells, tachyzoites exhibited consistently more [3H]-uracil incorporation when incubated with retinal cells than when incubated with the other cell types. The absolute values obtained for each cell line varied between assays. However, the relationships between samples were consistent. On average, trichloroacetic-acid-insoluble radioactivity for tachyzoite-retinal endothelial cultures was 55% higher than that measured for the tachyzoite-aortic endothelial cultures, and 35% higher than that measured for the tachyzoite-umbilical vein endothelial cell cultures. In three of four assays, the differences between radioactivity measured for retinal versus other endothelial cell cultures were statistically significant (assay 1: P < 0.001 and 0.002; assay 2: P = 0.020 and 0.003; and assay 3: P = 0.005 and 0.007, for retinal versus aortic and umbilical vein endothelium, respectively). In a fourth assay, a trend toward higher radioactivity by tachyzoite-retinal cell cultures was observed. The comparison between these cells and the other endothelial types was not quite significant (assay 4: P = 0.070 and 0.065, for retinal versus aorta and umbilical vein endothelium, respectively). A graph of a representative assay (assay 1) is provided in Figure 1.

When trichloroacetic-acid-insoluble radioactivity was compared for tachyzoites incubated with dermal versus retinal microvasculature endothelial cultures, significantly higher levels were measured for retinal cells in four of four independent assays (assay 1: P = 0.005; assay 2: P = 0.002; assay 3: P = 0.016; and assay 4: P = 0.045 in the four assays). As discussed in the preceding paragraph, absolute values varied between assays, but retinal cell cultures yielded readings that were 33% higher on average than dermal cells. Figure 2 shows a representative assay (assay 3).

In contrast to the differences observed between retinal endothelial cells and different retinal cell subtypes, when retinal microvascular endothelial cells from different donors were compared for tachyzoite proliferation using the [3H]-uracil-incorporation assay, although absolute values for each line varied between assays, there were no significant differences between cells from any of the three donors in four of five assays (P > 0.05). In one assay, tachyzoite incubation with cells from donor 2 yielded slightly higher (0% more) [3H]-uracil incorporation than cells from donor 3, and this difference was statistically significant (P = 0.038). However, a similar difference between cells from donors 2 and 3 was not observed in the other four assays. A representative assay is shown graphically in Figure 3.

In all assays, uninfected negative control wells of each cell subtype yielded minimal radioactivity levels, and wells contain-
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RESULTS

Results from this study show that retinal microvascular endothelium is preferentially susceptible to infection with T. gondii tachyzoites in comparison to selected other vascular endothelial cell subtypes, as measured by [3H]-uracil uptake by the organism in cell culture. Retinal endothelial-tachyzoite cultures exhibited between approximately 30% and 50% more trichloroacetic acid-insoluble radioactivity than tachyzoite-dermal microvascular endothelial cultures, a difference that was greatest in the case of retinal endothelium. Thus, across the entire series of experiments, tachyzoites cultured with cells from donor 1, which was used as the retinal cell line in all assays comparing different endothelial cell subtypes, as well as the three donor retinal endothelial cell assays, showed on average 2.78 higher trichloroacetic acid-insoluble radioactivity than tachyzoites cultured with foreskin fibroblasts.

DISCUSSION

Previous work with nonendothelial cell populations supports the concept of differential susceptibility of different cell types to T. gondii infection. In one study, which involved scoring cells for numbers of parasites per vacuole, a difference in parasite number was observed between monocytes and dendritic cells, which were relatively susceptible to infection, and other leukocyte subtypes (i.e., neutrophils and lymphocytes), which were relatively resistant. In an independent study of the brain which used a similar method to determine infectivity, astrocytes and neurons were more susceptible to tachyzoite infection than microglia. In 2000, Channon et al. introduced the concept of “permissive” versus “nonpermissive” (to rapid tachyzoite replication) host cells. They classified the foreskin fibroblast as a “permissive” host, highlighting that

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retinal vascular endothelium is highly susceptible to infection with *T. gondii*; in comparison with retinal endothelial cells, we found that foreskin fibroblasts were relatively resistant to tachyzoite replication. In fact, our findings question the clinical applicability of the foreskin fibroblast cell culture system that is used in many studies of the mechanisms of *T. gondii* infection, although these cells do not support an infection in the clinical sense. It might be anticipated that susceptibility of a given cell subtype would vary between individuals with different genetic backgrounds. In the tachyzoite–three-donor retinal endothelial cell assay (Fig. 3), we saw remarkable consistency in cell behavior, although given the limited number of donors, it is difficult to exclude the possibility of interindividual variation, particularly in relation to single gene defects of low prevalence.

Although the [3H]-uracil incorporation provides evidence that *T. gondii* preferentially infects retinal vascular endothelium, it cannot differentiate among selective binding of tachyzoites to the retinal vascular endothelial surface, relative ease of penetration into the cell, high rate of replication within the cell and/or low cell survival as possible mechanistic explanations. Microneme proteins contain sequences that are homologous to adhesive domains from vertebrate hosts, implying that tachyzoites exploit as yet unidentified surface receptor(s), which may have differential distribution, to gain entry.\(^{24}\) Cell morphology, including the presence or absence of fenestrations, may also impact tachyzoite entry into a cell. The level of host cell cholesterol controls formation of the parasitophorous vacuole.\(^{25}\) An intracellular environment that is low in reactive oxygen free radicals or reactive nitrogen metabolites and/or high in iron or tryptophan is known to facilitate tachyzoite replication.\(^{13}\)

We have demonstrated a relatively high susceptibility of cultured retinal microvascular endothelium to *T. gondii* infection in comparison to a number of other endothelial cell types. Our findings may explain, at least in part, the observed occurrence of an eye-selective disease, retinochoroiditis, after exposure to the organism. As recognized by others,\(^{30-31}\) the process of tissue culture may alter cell phenotype, obscuring or creating real differences between cell subtypes. To reduce this possibility, we used cells only in early passage. It is also worth noting the clear evidence that cells maintained in culture continue to show differences that reflect in vivo settings. For example, after culture, synoviocytes from a patient with rheumatoid arthritis continue to differ from synoviocytes obtained from a patient with osteoarthritis.\(^32\) Assays using animal models or fresh tissue may provide further confirmation of our findings. If tissue specificity of *T. gondii* infection is dependent on a selective interaction with the retinal microvasculature, targeted therapies that are directed toward this interaction may offer a new treatment option for toxoplasmic retinochoroiditis.

References


18. Discussion. International Conference on Toxoplasmosis, Copenhagen, Denmark, June 2005.


