Journal of Structural Biology 193 (2016) 162–171

Contents lists available at ScienceDirect

Journal of Structural Biology

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

Three-dimensional analysis of morphological changes in the malaria parasite infected red blood cell by serial block-face scanning electron microscopy

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ARTICLE INFO

Article history:
Received 6 October 2015
Received in revised form 16 December 2015
Accepted 5 January 2016
Available online 7 January 2016

Keywords:
Malaria
Plasmodium falciparum
Red blood cell
Whole cell imaging
Serial block-face SEM (SBF-SEM)

ABSTRACT

The human malaria parasite, *Plasmodium falciparum*, exhibits morphological changes during the blood stage cycle in vertebrate hosts. Here, we used serial block-face scanning electron microscopy (SBF-SEM) to visualize the entire structures of *P. falciparum*-infected red blood cells (iRBCs) and to examine their morphological and volumetric changes at different stages. During developmental stages, the parasite forms Maurer's clefts and vesicles in the iRBC cytoplasm and knobs on the iRBC surface, and extensively remodels the iRBC structure for proliferation of the parasite. In our observations, the Maurer's clefts and vesicles in the *P. falciparum*-iRBCs, resembling the so-called tubovesicular network (TVN), were not connected to each other, and continuous membrane networks were not observed between the parasitophorous vacuole membrane (PVM) and the iRBC cytoplasmic membrane. In the volumetric analysis, the iRBC volume initially increased and then decreased to the end of the blood stage cycle. This suggests that it is necessary to absorb a substantial amount of nutrients from outside the iRBC during the initial stage, but to release waste materials from inside the iRBC at the multinucleate stage. Transportation of the materials may be through the iRBC membrane, rather than a special structure formed by the parasite, because there is no direct connection between the iRBC membrane and the parasite. These results provide new insights as to how the malaria parasite grows in the iRBC and remodels iRBC structure during developmental stages; these observation can serve as a baseline for further experiments on the effects of therapeutic agents on malaria.

1. Introduction

Plasmodium parasites, the causative agents of malaria, have two distinct life cycles: one is a sexual cycle in mosquitoes and the other is an asexual cycle in vertebrate hosts. Furthermore, in both cycles the parasites exhibit several developmental stages with a distinctive morphology. During the blood stage cycle in vertebrate hosts, merozoites of *Plasmodium* invade red blood cells (RBCs) and develop into ring, trophozoite, and schizont stages within the parasitophorous vacuole (PV) surrounded by PV membrane (PVM) that form in the infected RBCs (iRBCs). At the schizont stage, daughter merozoites are produced and finally are released from the iRBCs into the bloodstream. During development in the iRBCs, the parasites extensively remodel the iRBC structures to permit replication. By exporting numerous proteins across the PVM into the iRBC cytoplasm and cytoplasmic membrane, it makes possible evasion of immune clearance. *Plasmodium falciparum*, that causes the most severe form of human malaria, forms membranous structures (Maurer's clefts) in the iRBC cytoplasm for protein sorting. Some exported proteins are situated at electron-dense protrusions, known as knobs, on the iRBC cytoplasmic membrane. Knob-associated histidine-rich protein (KAHRP) is a major knob component that interacts with RBC cytoskeletal proteins such as spectrin

Abbreviations: RBC, red blood cell; iRBC, infected RBC; SEM, scanning electron microscopy; SBF-SEM, serial block-face SEM; TVN, tubovesicular network; PVM, parasitophorous vacuole membrane; TEM, transmission electron microscopy.

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http://dx.doi.org/10.1016/j.jsb.2016.01.003
1047-8477/

and actin, and anchors *P. falciparum* erythrocyte membrane protein 1 (PfEMP1) to the knobs (Oh et al., 2000; Waller et al., 1999). The PfEMP1 is a major virulence factor, which binds to several receptors on the microvasculature (Sherman et al., 2003). In addition to these knob-associated proteins, other cytoskeletal-associated parasite proteins such as RESA, MESA and PfEMP3 also modify the structural properties of the iRBC and increase the rigidity and adhesiveness (Maier et al., 2009).

High-resolution 3D imaging of the *Plasmodium* parasite and the iRBC has made significant contributions to our understanding of the infection and replication mechanisms. Conventional transmission electron microscopy (TEM) using resin-embedded specimens has played an important role in the visualization of the parasite structures at each developmental stage in the iRBCs and the iRBC structures. Advanced cryo-electron microscopy enables the visualization of finer organelle structures in the parasites (Kudryashev et al., 2010; Lemgruber et al., 2013). Serial thin-section images obtained by TEM visualized subcellular structures, such as merozoite bud in the schizont (Bannister et al., 2003), apicoplast (Hopkins et al., 1999), mitochondrion (Slomianny and Prensier, 1986), and Maurer's clefts (Wickert et al., 2004), and detailed representations of each parasite stage were introduced (Bannister et al., 2000). Electron tomography using a thick section showed 3D shapes of Maurer's clefts and tether-like structures in Equinatoxin II (EqtII)-permeabilized iRBCs (Hanssen et al., 2008). On the other hand, to understand how the malaria parasite grows in the iRBC and remodels the iRBC structure during developmental stages, 3D structure of the entire parasite in the iRBC at each stage is required. However, numerous serial thin-section images by TEM or electron tomography based on serial thick sections (Abu Bakar et al., 2010; Hanssen et al., 2010a,b) or X-ray tomography (Hanssen et al., 2011, 2012, 2013; Kapishnikov et al., 2012) are necessary to reconstruct the 3D structure of the whole parasite; this requires high technical skills and efforts. For these reasons, it has been difficult to examine quantitatively the morphological changes of the *Plasmodium* parasite during the life cycle by these methods.

Recently, two serial block-face imaging techniques using scanning electron microscopy (SEM) have been developed; these enable much more efficient data acquisition of serial section images than the methods described above. In these new methods, the thin surface of a resin-embedded specimen is removed by a focused gallium ion beam; this method is called focused ion beam scanning electron microscopy (FIB-SEM; Heymann et al., 2006; Knott et al., 2008), or by a diamond knife attached to an in-chamber ultramicrotome; this method is called serial block-face scanning electron microscopy (SBF-SEM; Denk and Horstmann, 2004). The newly exposed surface structure is then imaged by SEM. The surface removal and imaging are automatically repeated to get serial block-face images of the specimen. While FIB-SEM has been applied to visualize 3D subcellular structures within the entire iRBC (Kapishnikov et al., 2012; Weiner et al., 2011; Medeiros et al., 2012; Kan et al., 2014), SBF-SEM has never been applied for the structural analysis of malaria parasites, which is preferred to collect massive data set from a large field of view and makes possible a volumetric analysis of whole cells and organelles.

In this study, we reconstructed 3D structures of entire *P. falciparum*-iRBCs at different developmental stages by SBF-SEM, and performed quantitative structural analysis to examine morphological changes of the cells and components during development. This analysis revealed that the volume of iRBC changed according to the growth of the parasite, although there was no structure directly connecting the parasite to the iRBC membrane, suggesting that the mass transportation between the outside the iRBC and the parasite was via the iRBC membrane transport systems such as parasite-induced new permeation pathways (Ginsburg and Stein, 1987; Kirk, 2001). Here we report for the first time volumetric changes at the ultrastructural level of cell components including mitochondrion and apicoplast with respect to each developmental stage. These whole cell data provide new insights as to how the malaria parasite grows and remodels the iRBC structure during development.

2. Materials and methods

2.1. P. falciparum culture

P. falciparum MS822 was isolated in Mae Sot, Thailand in 1988 and recently culture-adapted at the Institute of Tropical Medicine, Nagasaki University (Nakazawa et al., 2011). The parasites were cultured as described previously (Trager and Jensen, 1976), with human O+ RBCs in RPMI1640 (Invitrogen, Carlsbad, CA, USA) medium supplemented with 5% heat-inactivated pooled type AB^+ human serum, 5 mg/ml AlbuMax I (Invitrogen), 200 mM hypoxanthine (Sigma–Aldrich, St Louis, MO, USA), 0.23% sodium bicarbonate and 10 μg/ml gentamycin (Invitrogen). The cultures were maintained at 2% haematocrit and the levels of parasitaemia were monitored by examining Giemsa-stained blood smears until they reached ~20%.

2.2. Sample preparation for SBF-SEM

Samples for SBF-SEM were prepared as described previously (Deerinck et al., 2010). *P. falciparum*-iRBCs were collected and fixed with 2% glutaraldehyde in 0.1 M sodium cacodylate buffer containing 1 mM $CaCl_2$ and 1 mM $MgCl_2$ (cacodylate buffer, pH 7.4) for one hour at 4 °C. The iRBCs were rinsed five times with cacodylate buffer (each for 3 min), and then post-fixed for one hour at 4 °C with 1% osmium tetroxide and 1.5% potassium ferrocyanide in cacodylate buffer. The cells were washed with distilled water and incubated in 1% thiocarbohydrazide solution for 20 min at room temperature. They were then washed with distilled water, and fixed again with 1% osmium tetroxide in distilled water for 30 min at room temperature. After washes with distilled water, the samples were stained *en bloc* with 1% uranyl acetate in 75% ethanol overnight at 4 °C. After again washing with distilled water, they were stained with lead citrate (Reynolds, 1963) for 30 min at 4 °C. After another distilled water rinse, the cells were dehydrated through a series of ascending concentration of ethanol and acetone, and embedded in Quetol 651 epoxy resin (Nisshin EM, Tokyo, Japan). To confirm morphological preservation and *en bloc* heavy metal staining, ultrathin sections were prepared, and observed by TEM (JEM-1230; JEOL, Tokyo, Japan) without further heavy metal staining. We obtained enhanced membrane contrast in every sample of *P. falciparum*-iRBCs using this staining method, and the method was also reproducible for other *Plasmodium* species, *P. yoelii* and *P. knowlesi* (unpublished data).

2.3. Data acquisition using SBF-SEM

SBF-SEM images were acquired as reported previously (Miyazaki et al., 2014). The resin-embedded samples were trimmed and glued on top of aluminum rivets with conductive epoxy resin (SPI Conductive Silver Epoxy; SPI Supplies and Structure Probe, West Chester, PA, USA). The sides of the samples were coated with colloidal silver paint (Electron Microscopy Sciences, Hatfield, PA, USA), and the samples were placed into a FE-SEM (MERLIN; Carl Zeiss Microscopy, Jena, Germany) equipped with an in-chamber ultramicrotome system (3View; Gatan, Pleasanton, CA, USA). The block surface was thin sliced by an ultramicrotome