POTENTIAL CLINICAL RELEVANCE

Nanomedicine: Nanotechnology, Biology, and Medicine
8 (2012) 17–19

Short Communication

nanomedjournal.com

Infection by *Plasmodium* changes shape and stiffness of hepatic cells

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Received 1 July 2011; accepted 4 October 2011

Abstract

Infection of liver cells by *Plasmodium*, the malaria parasite, is a clinically silent, obligatory step of the parasite's life cycle. The authors studied the progression of *Plasmodium* infection in hepatic cells by atomic force microscopy, measuring both topographical and nanomechanical changes upon infection. In recent years, several studies have suggested that cellular nanomechanical properties can be correlated with disease progression. The authors' results show that infected cells exhibit considerable topographical changes, which can be correlated with the presence of the parasite, leading to a significant roughening of the cell membrane. The nanomechanical measurements showed that infected cells were significantly stiffer than noninfected cells. Furthermore, the stiffening of the cells appeared to be a cellular reaction to the *Plasmodium* infection, rather than a result of the stiffness of the invading parasites themselves. This article provides the first evidence of mechanical changes occurring in hepatic cells in response to *Plasmodium* infection.

From the Clinical Editor: The authors have studied the progression of *Plasmodium* infection in hepatic cells by atomic force microscopy, measuring topographical and nanomechanical changes upon infection. The nanomechanical measurements demonstrated that infected cells were significantly stiffer than noninfected cells.

Key words: Malaria; *Plasmodium*; Atomic force microscopy; Nanoindentation; Liver; Hepatic cells

Malaria, caused by the protozoan parasite *Plasmodium*, still imposes a horrific public health burden on large areas of the world. In the mammalian host, *Plasmodium* sporozoites deposited in the skin by *Anopheles* mosquitoes travel to the liver, infecting hepatocytes with formation of a parasitophorous vacuole (PV), where they develop into exoerythrocytic forms (EEFs) and multiply to generate thousands of merozoites, later released into the bloodstream and causing disease.[1] Although clinically silent, the liver stage is an obligatory step for *Plasmodium* infection and, therefore, a crucial determinant of disease progression. The intense replication process at this stage is probably accompanied by a subversion of the hepatocyte resources ensuring the parasite's survival.[2,3] However, nothing is known about the nanomechanical properties of infected hepatocytes or how they are affected by EEF growth. Increasingly, atomic force microscopy (AFM) has proven useful to characterize a range of pathogens and cells under pathological conditions, both in terms of topographic and nanomechanical properties.[4,5] Here, using AFM-based nanomechanical measurements, we report on the considerable physical changes that occur in both topography and mechanical properties of the hepatic cells upon *Plasmodium* infection and speculate on the molecular bases of these alterations.

Methods

We used an in vitro malaria infection system that employs Huh7 cells, a human hepatoma cell line, and the rodent model parasite *Plasmodium berghei* ANKA.[6] In this system, EEFs can be detected inside hepatoma cells until ~72 hours after invasion (hpi).[2] The use of GFP-expressing parasites enabled the definitive identification of infected cells within a monolayer by fluorescence microscopy. AFM imaging experiments were conducted on fixed, dried cells in air. AFM-based nanoindentation experiments were carried out in liquid at 37°C on living cells. Additional experimental details are included in the Supplementary Materials.

Results

To determine whether any changes occur in the morphology of hepatic cells upon *Plasmodium* infection, we initially

The authors declare that there is no conflict of interest.

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doi:10.1016/j.nano.2011.10.004

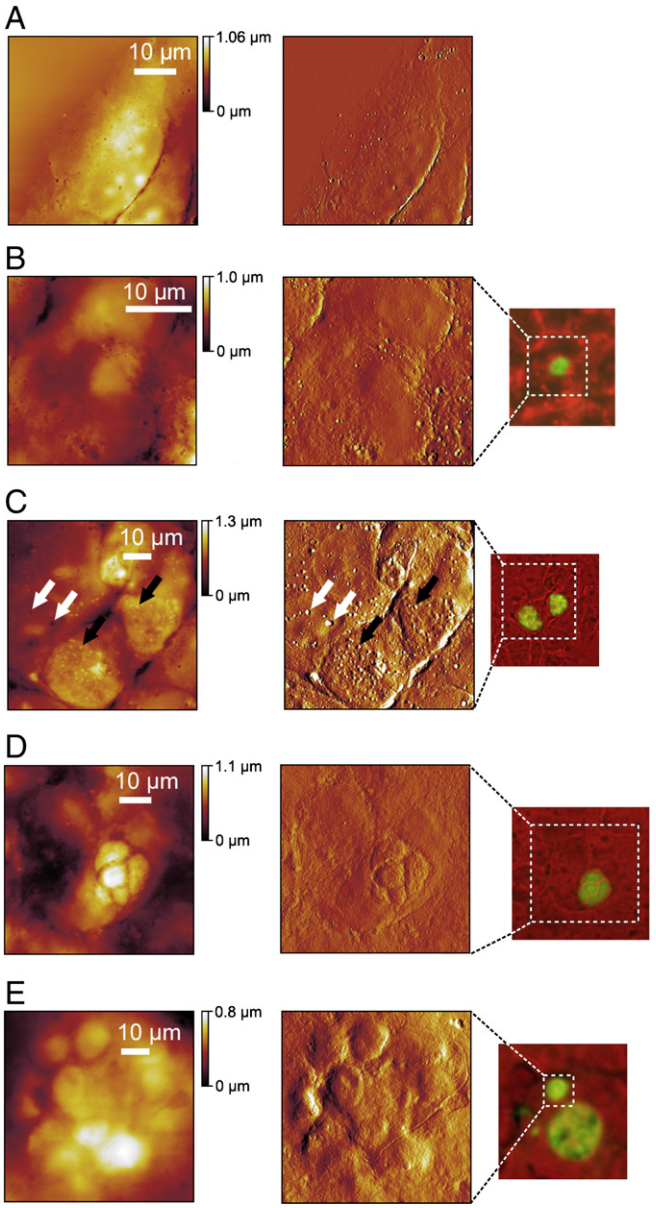

Figure 1. Representative images of dried and fixed hepatoma cells. **(A)** AFM image of noninfected cell (left: height image; right: amplitude image). (**B, C, D** and **E**) AFM height (left) and amplitude (center), with corresponding epifluorescence (right) images of infected cells fixed 24 **(B)**, 48 **(C)** 72 **(D)** and 72 **(E)** hpi with GFP-expressing *P. berghei* parasites (phase contrast images of cells are red channel, GFP channel is green). The white arrows in **C** highlight lowered features in noninfected cells and the black arrows raised features above the location of EEFs.

conducted imaging experiments on fixed cells. Considerable changes were visible in the infected cells in comparison with noninfected cells (Figure 1). These changes included the observation of growth of the PV, which was typically seen as a large, rounded feature raised above the main body of the cell, as well as changes in membrane texture. The changes in texture took the form of alteration in the numbers of smaller raised and lowered features on the surface and could be discriminated by measuring changes in membrane roughness. The measurements

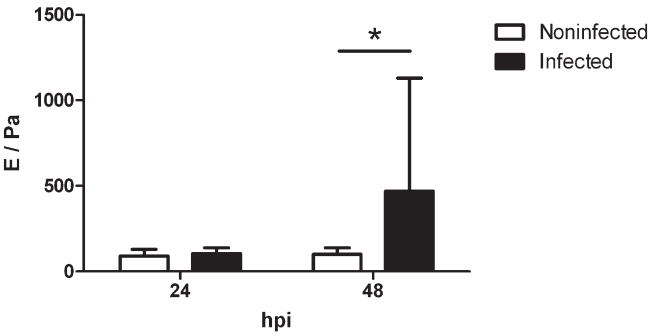

Figure 2. Overall nanoindentation results. The nanoindentation data are presented as Young's Modulus **(E)**, expressed in Pascals, and are the results of modeling as described in the text and supplementary materials. The error bars represent the standard deviation of the plotted means. Starred data pairs are significantly different ($P < 0.05$).

of membrane texture are described in the Supplementary Materials. These AFM imaging studies suggest that parasite development alters the topography of the host cell.

To test whether this could be related to physical changes that occur within the cell, we performed indentation analysis on cells at 24 and 48 hpi (Figure 2). Although no significant differences could be detected between the infected and noninfected cells at 24 hpi, infected cells at 48 hpi displayed significantly higher Young's modulus (E) values, and as such greater stiffness, relative to noninfected cells (4.7× ; $P < 0.0001$; Figure 2).

In addition, we made measurements in selected regions of the infected cells over the area containing the EEFs (as revealed by GFP fluorescence) or on a region of the membrane away from the parasitophorous vacuole. That data showed considerable differences between different regions on the infected cells. At 24 hpi, the membrane above the infected cell body was 1.36× stiffer than the area above the EEF ($P = 0.007$). This difference increased significantly from 24 hpi to 48 hpi (3.88×; $P < 0.0001$) (Figure 3).

Discussion

Intense *Plasmodium* replication inside hepatic cells is associated with a coordinated and sequential set of biological alterations in the host cell[3] including changes in its permeability.[2] However, whether these alterations have any impact in the nanomechanical properties of infected hepatocytes remains to be established. The data are the first evidence of the mechanical pressure exerted by *Plasmodium* PV growth on liver cells during infection. Indeed, using AFM-based nanomechanical measurements, we report: (i) morphological and topographical differences between infected and noninfected cells; (ii) increased stiffness in *P. berghei* infected hepatoma cells, specially at 48 hours after infection; and (iii) increased stiffness in infected cells, occurring in the membrane area over the main body of the cell, rather than directly over the area where the EEFs grew.

The large variation of the nanoindentation results at 48 hpi might be due to the heterogeneity in infection, with some parasites being more advanced in their development than others

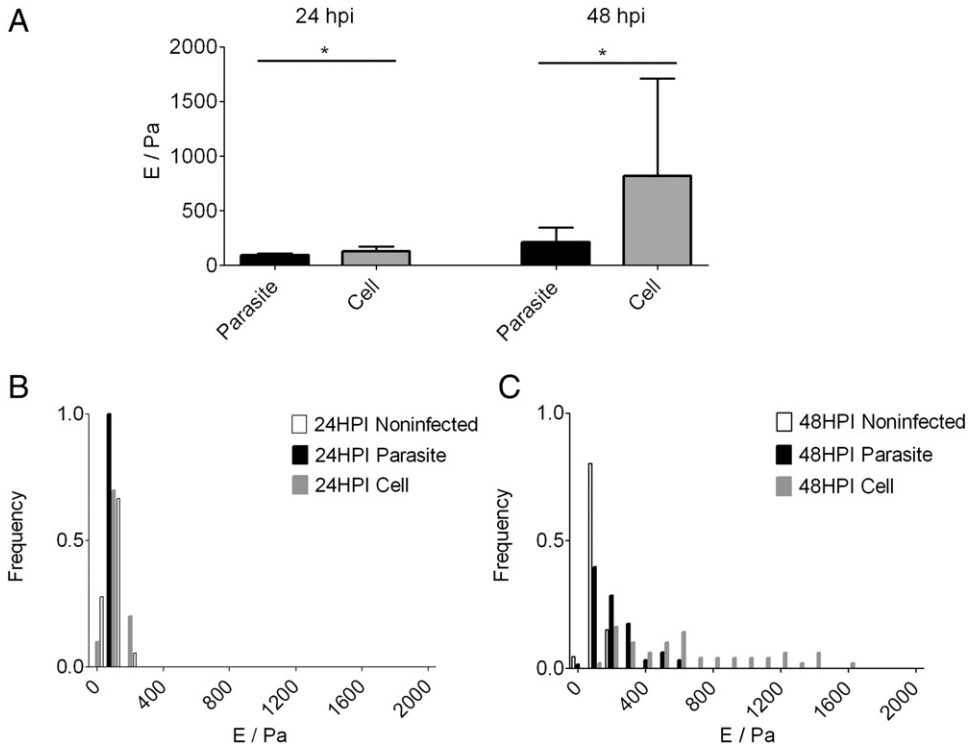

Figure 3. **(A)** Results of nanoindentation comparing results on infected cells measured directly over the parasite location (EEF) and over the main body of the cell. Starred pairs were significantly different ($P < 0.05$). **(B and C)** histograms illustrating the distribution of values obtained by nanoindentation measurements on cells 24 **(B)** and 48 **(C)** hpi.

were. Cell stiffness and structure depend mainly on the three components that collectively make up the cytoskeleton (i.e., microtubules, intermediate filaments, and actin filaments), which vary greatly in their elastic moduli and dimensions, and which can be quite heterogeneously distributed throughout the cell at the nanoscale.[4,7] We propose that, when applying nanoindentation experiments, some results would have been obtained directly over cytoskeleton fibers close to the membrane surface, explaining the very large variation in all the nanoindentation results. Therefore, these results suggest that the stiffening in the cell may occur in the cytoskeletal components.

Altogether, the work presented here underlines the great potential of AFM as a nanotechnology-based tool for unraveling cellular and subcellular details in disease progression, not just in terms of topography but also in terms of nanomechanical properties. More important, it reveals the impact that *Plasmodium* development and replication inside hepatic cells have on the nanomechanical properties of the infected cells, as previously shown for *Plasmodium*-infected red blood cells.[8] Whether these changes have any consequence in the function of those cells and how that might impact on pathogenesis remain to be investigated.

Acknowledgments

The authors would thank Ana Serra-Caetano for her assistance with the fluorescence activated cell sorting procedure and Maria José Feio for help with the figures.

Appendix A. Supplementary data

Supplementary data to this article can be found online at doi:10.1016/j.nano.2011.10.004.

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