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Osteoclastogenesis induced by CHIKV-infected fibroblast-like synoviocytes: A possible interplay between synoviocytes and monocytes/macrophages in CHIKV-induced arthralgia/arthritis

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ABSTRACT

Fibroblast-like synoviocytes are known to migrate from joint to joint and are proposed to be one of the key players in the inflammatory cascade amplification in rheumatoid arthritis patients. In the recent CHIKV epidemic, patients developed arthritis-like syndrome and the synoviocyte is one of the suspected players in CHIKV-induced polyarthritis. Thus, to learn more on this syndrome, the responses of fibroblast-like synoviocytes to chikungunya virus (CHIKV) infection, and the interaction between CHIKV-infected synoviocytes and phagocytes, were investigated. Primary human fibroblast-like synoviocyte (HFLS) cultures were infected with clinical isolates of CHIKV at an MOI of 0.001 pfu/cell. Data indicated that HFLS are permissive to CHIKV replication, generating peak titers of 10^5–10^6 pfu/ml. Interestingly, CHIKV-infected HFLS cultures secreted mainly the mediators that are responsible for phagocytes recruitment and differentiation (RANKL, IL-6, IL-8 and MCP-1) but not arthritogenic mediators (TNF-α, IL-1β, MMP-1, MMP-2 or MMP-13). The interaction between CHIKV-infected synoviocytes and phagocytes was studied using UV-irradiated, CHIKV-infected HFLS supernatant. Data revealed that supernatants from CHIKV-infected HFLS cultures not only induced migration of primary human monocytes, but also drove monocytes/macrophages into osteoclast-like cells. These differentiated osteoclast-like cells produced high levels of TNF-α and IL-6, principal mediators of arthritis. This data suggests a potential interplay between infected HFLS and recruiting phagocytes which may responsible for the arthralgia/arthritis in CHIKV-infected patients.

1. Introduction

Chikungunya virus (CHIKV) belongs to the genus *Alphavirus* of the family Togaviridae. Its genome is a linear single-strand, message-sense RNA with a length of ~11.8 kb which has two open reading frames (ORF). The ORF at the 5′ end codes for a non-structural polyprotein which serves as the precursor of the non-structural proteins 1–4 (nsP1-4) while the second ORF encodes the structural proteins which are capsid (C) and envelope proteins (E1, E2) as well as two small peptides, E3 and 6 K (Chevillon et al., 2008). CHIKV causes a disease called Chikungunya fever which was first formally reported after an outbreak in Tanzania in 1952 (Robinson, 1955) and the causative agent was subsequently isolated from the same outbreak (Ross, 1956). Since the first reported epidemic, CHIKV caused numerous outbreaks in Africa and Asia but it was neglected because of its generally benign nature (Ali Ou Alla and Combe, 2011; Burt et al., 2012). Starting from 2004/2005 CHIKV suddenly re-emerged leading to a worldwide epidemic affecting nearly 40 countries in Southeast Asia, the Pacific region and Europe with several million estimated cases. Of particular concern was the fact that the re-emerged CHIKV not only caused significant economic loss due to the high number of incapacitated arthralgia/arthritis patients, but also the occurrence of morbidities and fatalities (Gerardin et al., 2011; Labeaud et al., 2011; Pialoux et al., 2007; Soumahoro et al., 2011). This markedly increased local and international awareness of the disease.

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Most individuals infected with CHIKV develop symptoms which have two consecutive phases (Staples et al., 2009). The initial phase usually presents as a high fever with headache, skin rash, myalgia, and severe arthralgia in the small joints of the hands and toes. These symptoms are typically cleared within a few days or a couple of weeks. In some cases the disease can progress to a second phase which is characterized by a chronic rheumatic syndrome (Schilte et al., 2013). Although Chikungunya fever is usually not fatal, its severity is due to the severe arthralgia that may persist for weeks or years, and the joint pain is real burden because of its serious negative impact on the individual and on the affected communities (Gerardin et al., 2011; Labeaud et al., 2011). Thus, understanding the mechanism of CHIKV-induced chronic arthritis is important to understanding the pathology of the disease. A large body of evidence suggests that age, underlying medical conditions, viral load and host responses are factors predisposing to a more severe CHIKV presentation and long-term sequelae (Hoarau et al., 2010; Sissoko et al., 2009; Staikowsky et al., 2009). Among these factors, the role of host immune response has been intensively studied, and the association between vascular proinflammatory cytokines/chemokines and CHIKV-associated arthralgia/arthritis has been investigated by several groups. Interestingly, most of the reports emphasize a link between IL-1β and IL-6 in severity during the acute phase of the disease, while elevation of IL-6, MCP-1 and IL-8 are associated with chronic phase or prolonged arthralgia (Chow et al., 2011; Dupuis-Maguiraga et al., 2012; Lohachanakul et al., 2012; Ng et al., 2009; Win et al., 2010). IL-6 serves as a potent endogenous pyrogen and the activator of the joint pain through sensitizing joint nociceptors and also participates in inflammation-evoked pain through neurons (Schaible et al., 2010). Beside soluble mediators, immune cells, dendritic cells, monocytes/macrophages and $CD4^+$ T cells have been shown to regulate chikungunya virus pathogenesis in *in vivo* models (Long et al., 2013; Rulli et al., 2011; Teo et al., 2013).

Since arthralgia/arthritis is a joint pathology, understanding the mechanism of CHIKV induced arthralgia/arthritis requires greater understanding of the interaction between cells of the joint and CHIKV. Joints are generally classed as fibrous, cartilaginous, or synovial, and it is this last class of joint that is involved in arthritis/arthralgia (Komatsu and Takayanagi, 2012). The synovial membrane or synovium is the smooth lining of the synovial joint and this membrane consists of two layers, the sublining and the intimal lining which is in contact with the joint cavity and produces synovial fluid (Bartok and Firestein, 2010). The intimal layer consists of fibroblast like synoviocytes (FLS) and macrophage like synovial cells (Bartok and Firestein, 2010). FLS play a key role in inflammatory cascade amplification, bridging innate and adaptive immunity, and FLS are known to be a central mediator of joint damage in inflammatory arthritides of either infectious or noninfectious origins (Bartok and Firestein, 2010). FLS function through the production of matrix metalloproteinases that degrade collagen and *via* activation of osteoclasts to attack bone (Cooles and Isaacs, 2011). The synovium is suspected to be a target for CHIKV (Hoarau et al., 2010) and therefore information on the interaction between CHIKV and the synovium may facilitate our understanding on pathophysiology of CHIKV-induced arthralgia/arthritis. In the present study, the profile of arthritis-related mediators released from the CHIKV-infected primary human FLS was monitored at various time points of infection. In addition, the possible interplay between infected HFLS and monocyte/macrophages *via* mediators released from CHIKV-infected HFLS was investigated. We found that HFLS replicated CHIKV and secreted mediators that are able to recruit and differentiate monocytes/macrophages into osteoclasts. These differentiated osteoclasts produced high level of arthritogenic cytokines.

2. Materials and methods

2.1. Clinical CHIKV isolates

Chikungunya viruses used in this study were isolated from patients who presented to the Community Medical Unit, Pang Nga Hospital, Pang Nga, Thailand as previously described (Lohachanakul et al., 2012). This work was conducted according to the principles expressed in the Declaration of Helsinki. The patients were enrolled after informed consent was obtained. The study protocol was approved by Mahidol University International Review Board (COA.NO.MU-IRB 2010/251.3018) and by the Ethics Review Board of Pang Nga Hospital. Eight isolates obtained from patients enrolled in the above cohort were used in the present work. Four out of eight viruses, or severe isolates, were isolated from severe Chikungunya fever (CHIKF) patients. These patients presented with a fever of $\geq$38.5 °C, a pulse rate of $\geq$ 100 beats/min and a platelet count of $\leq 100 \times 10^9$ cells/L on the first day of clinical assessment. The other four isolates which are called mild isolates were viruses from mild CHIKF patients which were patients who presented with a fever of $\geq$37 °C to $\leq$38.5 °C, a pulse rate of $\leq$ 100 beats/min and a platelet count of $\geq 100 \times 10^9$ cells/L on the first day of hospital visit. Viruses were isolated from patient's sera using C6/36 cells. The working seeds were viruses of the 2nd passage in C6/36 cells. The amounts of CHIKV in those working stocks were quantified using plaque assay on Vero (ATCC Cat No. CCL-81) cells and were found to be in the range of 1×10^5–5×10^6 pfu/ml dependent on isolate.

2.2. UV-inactivation of viruses

The supernatant of CHIKV-infected HFLS containing of 10^5–10^6 pfu/ml, was irradiated in a lid-less 3 cm diameter culture dish, using a 30 W UV lamp at a distance of 10 cm for 90 min, at room temperature. Virus inactivation was confirmed by plaque assay.

2.3. Primary cell culture

Primary human fibroblast-like synoviocyte (HFLS) cells were obtained from Cell Applications, Inc. (San Diego, CA). Cells were isolated from the synovium of a 60 year old healthy man. The synoviocytes were characterized by their fibroblast-like morphology, growth pattern and the expression of CD55 on cell surface. The cells were cultured in synoviocyte growth medium (Cell Application, Inc.) at 37 °C in a 5% CO_2 humidified incubator. The culture medium was changed every alternate day and cells were sub-cultured when the culture reached 80% confluence.

2.4. Infection of HFLS cultures

1×10^5 HFLS cells were seeded into 12-well plates and cultured for 24 h before being inoculated separately with 4 mild- and 4 severe- CHIKV isolates. Briefly, monolayer cultures of HFLS were washed once with medium without fetal bovine serum and inoculated with 200 μl of CHIKV at an MOI of 0.001 pfu/cell, at 37 °C for 90 min. The inoculated cultures were then washed twice to remove unbound viruses and further cultured in 1.5 ml of culture medium with 2% FBS at 37 °C in a 5% CO_2 atmosphere. An aliquot of 200 μl of supernatant fluid was harvested every 24 h for five consecutive days and cultures were replenished with the same volume of fresh medium. The harvested supernatants were subjected to plaque assay for determination of the efficiency of viral replication. Inoculation of HFLS with the diluent was used as the control system.