Virus Research 179 (2014) 153–160

Contents lists available at ScienceDirect

Virus Research

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

Human pathogenic viruses are retained in and released by *Candida albicans* biofilm *in vitro*

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ARTICLE INFO

Article history:
Received 1 May 2013
Received in revised form 22 October 2013
Accepted 22 October 2013
Available online 30 October 2013

Keywords:
Biofilm
Candida albicans
Human Herpesvirus type-1 (HSV-1)
Coxsackievirus B5 (CVB5)

ABSTRACT

Candida albicans is the most prevalent human fungal pathogen associated with biofilm formation on indwelling medical devices. Under this form, Candida represents an infectious reservoir difficult to eradicate and possibly responsible for systemic, often lethal infections. Currently, no information is available on the occurrence and persistence of pathogenic viruses within *C. albicans* biofilm. Therefore, the aim of this study was to investigate whether *Herpes Simplex Virus type 1* (HSV-1) and *Coxsackievirus type* B5 (CVB5) can be encompassed in Candida biofilm, retain their infectivity and then be released. Thus, cell-free virus inocula or HSV-1-infected cells were added to 24 h-old fungal biofilm in tissue culture plates; 48 h later, the biofilm was detached by washing and energetic scratching and the presence of virus in the rescued material was end-point titrated on VERO cells. Planktonic Candida cultures and samples containing only medium were run in parallel as controls. We found that both HSV-1 and CVB5 free virus particles, as well as HSV-1 infected cells remain embedded in the biofilm retaining their infectivity. As a second step, the influence of biofilm on virus sensitivity to sodium hypochlorite and to specific neutralizing antibodies was investigated. The results showed that virus encompassment in fungal biofilm reduces virus sensitivity to chemical inactivation but does not affect antibody neutralization. Overall, these data provide the first *in vitro* evidence that viruses can be encompassed within Candida biofilm and then be released. Thus, it may be speculated that Candida biofilm can be a reservoir of viruses too, posing a further health risk.

1. Introduction

Biofilms are structured communities of microorganisms in which the cells bind to a surface and become embedded in a self-produced matrix of extracellular polymeric substances (Costerton et al., 1987; Hall-Stoodley et al., 2004; Lynch and Robertson, 2006; Flemming and Wingender, 2010). Biotic as well as abiotic surfaces, including mucosal tissues, indwelling medical devices, water piping systems or natural aquatic eco-systems, may all provide a useful support for biofilm building (Donlan, 2002).

Clinically, biofilm production accounts for up to approximately 65% of microbial infections (Donlan, 2001; Douglas, 2002; Hall-Stoodley et al., 2012). Most of them are implant-related infections, being located on the surface of devices such as catheters, prostheses, and heart valves (Donlan, 2001; Douglas, 2002, 2003). Also, about 50% of nosocomial infections are associated with medical devices (Kojic and Darouiche, 2004; Cauda, 2009); notoriously, such infections are difficult to be treated and often device removal becomes mandatory (Chandra et al., 2001; Donlan, 2001, 2002; Douglas, 2002, 2003; Kojic and Darouiche, 2004; Kumamoto and Vinces, 2005; Cauda, 2009). Overall, biofilm-related infections are a common complication for many hospitalized subjects and they represent an additional significant cost for medical management of these patients.

Candida spp, especially *Candida albicans*, is known as one of the major agents of hospital-acquired infections worldwide causing both mucosal and deep seated infections associated with candidemia (Pfaller and Diekema, 2007). Recent data estimate that *C. albicans* ranks as the fourth most common cause of bloodstream infection and is responsible for a significantly increasing number of device-related infections. The ability of *C. albicans* to form biofilms on medical devices has a great impact on its pathogenicity and it is related to most cases of invasive candidiasis (IC) (Sutherland et al., 2004; Blankenship and Mitchell, 2006; Nett and Andes, 2006). IC

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http://dx.doi.org/10.1016/j.virusres.2013.10.018

is associated with a high mortality rate, which is estimated to be about 40% in the United States and raging from 30% to 50% in other countries. Besides, treatment costs for IC are very high. (Mermel et al., 2001; Ramage et al., 2005; Blankenship and Mitchell, 2006; Nett and Andes, 2006).

The development of Candida biofilm occurs in three phases over a period of 24–48 h (Hughes et al., 1998; Hanlon et al., 2001; Donlan, 2002). The initial phase begins when single yeast cells that adhere to an abiotic or a biotic surface. In a second phase, cells proliferate and begin to switch to their hyphal form. In the final step Candida cells produce an extracellular polymeric matrix encompassing a dense network of filamentous forms (pseudohyphae and hyphae) and yeast cells. During this late phase, single Candida cells or biofilm fragments can detach from the biofilm itself. It follows that biofilm-infected devices serve as reservoirs of Candida that, once released into the bloodstream, may gain access to distant places and cause deep-seated fungal infection.

Several studies describe the effect of phages against bacterial biofilms showing that they can provide impressive strategies as anti-biofilm agents for their ability to kill bacteria (Duran et al., 2003; Donlan, 2009). Furthermore, many phages induce production of depolymerases that hydrolyze extracellular polymers of biofilm (Hughes et al., 1998). Conversely, there are only few investigations on the interactions between human pathogenic viruses and biofilm and these are mainly focused on water biofilms. Enteroviruses have been detected in biofilms inside water pipelines, stably attached with high resistance to water flow pressure and disinfection. These viruses encompassed in biofilm represent a risk for human health as a source of waterborne infections (Quignon et al., 1997; Storey and Ashbolt, 2003; Skraber et al., 2005; Wingender, 2011; Wingender and Flemming, 2011). Currently, there are no studies on the interplay between viruses and biofilms produced in humans.

By the present *in vitro* study, we examined the interactions between *C. albicans* biofilm and two human pathogenic viruses, namely, Herpes Simplex Virus-type 1 (HSV-1) and Coxsackievirus type B5 (CVB5). We assessed whether free viral particles could be encompassed in Candida biofilm, retaining their infectivity, and possibly be released. We also investigated the influence of biofilm on the virus sensitivity to hypochlorite and to neutralizing antibodies, as well as the ability of Candida biofilm to harbour HSV-1-infected cells.

2. Materials and methods

2.1. Candida albicans strains

Two strains of *C. albicans* were used: namely the highly virulent, biofilm producer strain 50vr isolated from a clinical case of invasive fungal infection, highly virulent in an infection model in *Galleria mellonella* (Cirasola et al., 2013) and the agerminative low virulent strain PCa_2 as a planktonic biofilm non-producer strain (Kurtz et al., 1996).

2.2. Virus strains

HSV-1 and CVB5 were clinical isolates, identified by monoclonal antibodies (Cermelli, 2002 and Cermelli, 2009). Both strains were laboratory adapted through serial passages (>50) on VERO cells over many years. The virus inocula employed in the experiments consisted of cell-free virus suspensions, obtained from centrifuged lysates of virus-infected VERO cells.

2.3. Cell lines

The epithelial cell line Vero, used to propagate and titrate HSV-1 and CVB5, was maintained in Eagle's Minimal Essential Medium (MEM) with 10% foetal calf serum (FCS), 2 mM L-glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin, at 37 °C and 5% CO_2. Additionally, the human T cell lymphoblast line JJHAN was used for experiments on the interaction between biofilm and cells in suspension. This cell line was maintained in RPMI 1640 added with same components as for MEM. Both cell lines were maintained by passages in fresh medium twice a week.

2.4. Biofilm formation and detachment

For biofilm production, Candida strain 50vr was grown overnight at 37 °C in Yeast Peptone Dextrose (YPD), harvested, washed with phosphate-buffered saline (PBS), and resuspended to 1×10^6 cells/ml in RPMI-10% FCS; 100 μl were seeded in duplicate in polystyrene, flat-bottom 96-well plates (Euroclone S.p.A., Pero (Mi), Italy) and incubated at 37 °C to allow biofilm formation, according to reported studies (Chandra et al., 2008; Pierce et al., 2008).

For biofilm detachment, 3 different methods were tested and compared.

First, a mechanical treatment: the culture wells were washed 3 times with 100 μl of PBS and then, after adding 50 μl of PBS, were energetically scraped for 2 min.

Second, an enzymatic detachment according to Al-Fattani and Douglas (2006): after 3 washings with 100 μl of PBS, the culture wells were added with 100 μl of proteinase K solution (50 μg/ml in Na_2HPO_4/NaH_2PO_4 buffer, pH 7.5) and the plates incubated for 2 additional hours at 37 °C. Afterwards, proteinase K was blocked by adding 50 μl of phenyl methane sulfonyl fluoride (25 mM) (Sigma–Aldrich Srl – Milan – Italy).

Third, a thermal detachment: the samples were washed 3 times with 100 μl of PBS and then, after adding 50 μl of PBS, the plate was heated at 55 °C for 15 min.

At the end of each detachment method, the rescued materials were transferred into microtubes, centrifuged at 14,000 rpm for 10 min and then the cell-free supernatants were processed for virus titration as detailed below.

2.5. XTT assay

The XTT colorimetric tetrazolium assay was used to determine fungal cell viability as an indirect quantification of Candida biofilm grown in culture wells as well as of biofilm remained in the wells after detachment (Kuhn et al., 2003). A commercial kit (AppliChem GmbH, Darmstadt, Germany) was employed following the manufacturer's instructions. Briefly, wells containing Candida biofilm, planktonic pCa2 Candida cells or controls (medium only) were washed 3 times with 200 μl of PBS and then 100 μl of the colorimetric solution were added: this solution contained 1 part of 2,3-bis (2-methoxy-4-nitro-5-sulfophenyl)-5-[(phenylamino) carbonyl]-2H-tetrazolium hydroxide (XTT) and 1 part of menadione 10 mM in acetone. After 2 h incubation in darkness at 37 °C the absorbance of the coloured reduction product was measured by a spectrophotometer (Sunrise, Tecan Group Ltd, Männedorf, Switzerland) at 450 nm. Each experimental group consisted of 6 replicates.

2.6. Crystal violet assay

Crystal Violet (CV) staining was used to quantify the biofilm grown in the presence of JJHAN cells (Jin et al., 2003): XXT was not used in this case since it stains all viable cells and does not allow to distinguish biofilm mass from JJHAN cells. Briefly, wells containing Candida biofilm, planktonic pCa2 Candida cells or controls (medium only) were washed 3 times with 200 μl of PBS and then air dried for 5′. After fixation by 100 μl of methanol for 20′,