Herpes simplex virus-1 entrapped in Candida albicans biofilm displays decreased sensitivity to antivirals and UVA1 laser treatment

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Abstract

**Background:** Recently, we published data suggesting a mutualistic relationship between HSV-1 and Candida albicans; in particular: (a) HSV-1 infected macrophages are inhibited in their anti-Candida effector function and (b) Candida biofilm protects HSV-1 from inactivation. The present in vitro study is aimed at testing the effects of Candida biofilm on HSV-1 sensitivity to pharmacological and physical stress, such as antiviral drugs (acyclovir and foscarnet) and laser UVA1 irradiation. We also investigated whether fungus growth pattern, either sessile or planktonic, influences HSV-1 sensitivity to antivirals.

**Methods:** Mature Candida biofilms were exposed to HSV-1 and then irradiated with laser light (UVA1, 355 λ). In another set of experiments, mature Candida biofilm were co-cultured with HSV-1 infected VERO cells in the presence of different concentrations of acyclovir or foscarnet. In both protocols, controls unexposed to laser or drugs were included. The viral yield of treated and untreated samples was evaluated by end-point titration. To evaluate whether this protective effect might occur in relation with a different growth pattern, HSV-1 infected cells were co-cultured with either sessile or planktonic forms of Candida and then assessed for susceptibility to antiviral drugs.

**Results:** UVA1 irradiation caused a 2 Log reduction of virus yield in the control cultures whereas the reduction was only 1 Log with Candida biofilm, regardless to the laser dose applied to the experimental samples (50 or 100 J/cm²). The presence of biofilm increased the IC₉₀ from 18.4–25.6 J/cm². Acyclovir caused a 2.3 Log reduction of virus yield in the control cultures whereas with Candida biofilm the reduction was only 0.5 Log; foscarnet determined a reduction of 1.4 Log in the controls and 0.2 Log in biofilm cultures. Consequently, the IC₅₀ for acyclovir and foscarnet increased by 4- and 12-folds, respectively, compared to controls. When HSV-1 was exposed to either sessile or planktonic fungal cells, the antiviral treatments caused approximately the same weak reduction of virus yield.

**Conclusions:** These data demonstrate that: (1) HSV-1 encompassed in Candida biofilm is protected from inactivation by physical (laser) and pharmacological (acyclovir or foscarnet) treatments; (2) the drug antiviral activity is reduced at a similar extent for both sessile or planktonic Candida.

**Keywords:** Biofilm, Candida albicans, Human herpesvirus type-1 (HSV-1), UVA, Acyclovir, Foscarnet, Laser, Virus

Background

In most natural environments, microorganisms exist predominantly as biofilms rather than as planktonic cells [1]. Growing as a biofilm provides microorganisms with a plethora of advantages. During biofilm formation, microorganisms characteristically display a phenotype that is markedly different from that of their planktonic counterpart, contributing to a reduced sensitivity to antimicrobial drugs and to host’s immune response [2–8]. In clinical setting, biofilm represents an ever-growing problem...
accounting for up to approximately 65% of infections, particularly severe in immunocompromised hosts [2].

Recently, _Candida albicans_ biofilm has gained prominence because of the increase in infections related to indwelling medical devices representing suitable surfaces for biofilm formation [9, 10]. These localized infectious foci can allow fungal cell detachment and dispersal, causing deep tissues candidiasis and candidemia, both associated with high mortality rates (30–50%) [11, 12]. The ability of _C. albicans_ to form biofilm has a great impact on its pathogenicity; given the dramatically increased resistance to antifungal agents, such as fluconazole and amphotericin, biofilm-related infections are difficult to eradicate [13].

It is reasonable to envisage that in vivo, especially in anatomical sites naturally harboring an abundant and complex resident microbiota, such biofilms likely occur as poly-microbial multi-layered network of different microorganisms, experiencing both synergistic and antagonistic relationships. Recently, by an in vitro model, we demonstrated that herpes simplex virus 1 (HSV-1) and coxsackie virus B5 can be efficiently entrapped in and protected by Candida biofilm [14]; moreover, literature reports show that HSV-1 enhances _C. albicans_ adherence, biofilm formation and resistance to host-mediated antifungal defenses [15, 16]. Such synergistic interactions between HSV-1 and _C. albicans_ provide the rationale for the present in vitro study aimed at testing the effects of fungal biofilm on virus biology in terms of sensitivity to antiviral drugs and laser irradiation. We also investigated whether fungus growth pattern, either sessile or planktonic, influences HSV-1 sensitivity to antivirals.

**Methods**

_Candida albicans_

The _C. albicans_ clinical isolate 50vr, used in the present study, was previously characterized as biofilm producer and highly virulent, as assessed by an in vivo infection model in _Galleria mellonella_ [17]. _C. albicans_ was kept in stock at −20 °C and maintained for experiments by bi-weekly passages on Sabouraud Dextrose Agar plates. Fresh cultures were set the day before each experiment.

**HSV-1**

The HSV-1 strain used in this work was a clinical isolate, identified by monoclonal antibodies, laboratory adapted through serial passages (> 50) on VERO cells [14, 15]. The virus inocula employed in the experiments consisted of cell-free virus suspensions, obtained from centrifuged lysates of virus-infected VERO cells. Virus batches were titrated on VERO cells (10⁶ PFU/mL) and kept frozen in aliquots at −80 °C.

**Cell line**

The VERO cell line was used for the all experiments. Cells were cultured at 37 °C and 5% CO₂ in minimum essential medium (MEM) with 10% (growth medium) or 5% (maintenance medium) foetal bovine serum (FBS), penicillin (100 U/mL), streptomycin (100 μg/mL), ciprofloxacin (100 μg/mL) and l-glutamine (2 mM). The cell line was maintained by passages in fresh medium twice a week.

**Laser source**

The Laser Alba 355 (Elettronica Valseriana, Casnigo, BG, Italy) was used as UVA1 laser source; it works at 355 λ, allowing to set different programs by combining parameters such as laser power, time of exposition, distance from the laser source and shape of the radiated area.

**Antiviral drugs**

Two antiviral molecules were assessed against HSV-1, acyclovir (Recordati SpA, Milano, Italy) and foscarnet (Clinigen, Burton-on-Trent, UK). Both were commercial products commonly used for intravenous treatment.

**Biofilm formation and exposure to HSV-1**

Candida cells were grown overnight at 37 °C in yeast peptone dextrose (YPD), then harvested and washed with phosphate-buffered saline (PBS). After resuspension to 1 × 10⁶ yeast cells/mL in MEM-10% FBS, 100 μL were seeded in duplicate in polystyrene, flat-bottom 96-well cell culture plates (Euroclone S.p.A., Pero-Mi, Italy) and incubated at 37 °C to allow biofilm formation, according to reported studies [14, 18, 19]. Twenty-four hours later, virus inoculum (50 μL, 10⁷ PFU/mL final concentration) was added to biofilm-containing wells and to empty control samples. The samples were incubated for additional 24 h and then exposed to either physical or pharmacological treatments. Finally, the wells were scraped for 1′ with a plastic tip and the load of infectious virus embedded in the detached/rescued biofilm was titrated (see below). Each experiment was repeated 3–4 times and each condition was tested in triplicate.

In a further set of experiments aimed at assessing whether drug antiviral efficacy may be different when Candida is cultivated as biofilm or as planktonic, Candida was also seeded in wells with a glass cover slide on the bottom. In our hands, glass inhibits the Candida strain we used (50vr) in biofilm formation: in fact, Candida 50vr when cultured on glass surfaces grow in a planktonic pattern (personal observation) with an hyphal mass floating in the culture medium.
Virus titration
In each experiment, HSV-1 residual titer was established by end-point titration. At the end of each experiment, plate well content was harvested by scraping for 1′ with a plastic tip. After centrifugation, the rescued material was diluted with maintenance medium on a tenfold basis and each dilution was seeded in duplicate onto 24 h-old VERO cell cultures. After a 3 day incubation at 37 °C, the virus titer of each sample was established as the highest dilution showing the typical viral cytopathic effect. The results, expressed as tissue culture infectious dose 50 (TCID$_{50}$/mL), were calculated using the Reed and Muench formula [20].

In order to determine the Inhibitory Concentration 90 (IC$_{90}$) or 50 (IC$_{50}$) of the different treatments, the plaque reduction assay was used and it was performed according to published procedures [21]. After centrifugation of the rescued material, VERO cell monolayers were infected with tenfold serial dilutions of such material. After 1 h of incubation at 37 °C, virus inoculum was removed and each well was added with maintenance medium containing human γ-globulin anti-HSV-1 at 0.6% to neutralize non-penetrated virus. Medium was removed 2 days later, and the infected cell monolayers were fixed with methanol and stained with crystal violet (CV) to count the cytolysis plaques: in this case, virus titer was expressed as plaque forming units (PFU/mL).

Laser treatment of C. albicans
The effects of UVA1 were evaluated on C. albicans to determine whether the laser energy could have an inhibitory activity on biofilm. In particular, to test the effects of the laser beam on biofilm formation, Candida cells were exposed to the laser beam immediately after seeding the yeast cells in culture medium (pre-treatment) and then plates were incubated for 48 h to allow biofilm formation. Also, the effects of laser irradiation were evaluated on biofilm maintenance, by exposing mature biofilm to laser beam, namely 48 h after cell seeding (post-treatment). Nine different emission protocols, with energy ranging between 20 and 250 J/cm$^2$, were applied. In any protocol tested, the laser beam had a square application and the amount of residual HSV-1 in supernatants of detached biofilm was determined by end-point titration on VERO cells. The same treatment was carried out also in culture wells without C. albicans (controls). The IC$_{90}$ of UVA1 treatment on HSV-1 was determined with or without Candida biofilm. Thus, after 24 h of incubation at 37 °C with virus, Candida biofilms were exposed or not to laser beam. Afterwards, washing and scraping for 1′ were performed and the load of infectious virus in the detached biofilm was determined by plaque assay on VERO cells. The same treatment was carried out also in culture wells without C. albicans (controls). The IC$_{90}$ of UVA1 treatment on HSV-1 was determined with or without Candida biofilm.

Assessment of virus sensitivity to laser treatment in the presence or absence of Candida biofilm
On the basis of XTT and CV assays results (see below), two laser protocols were employed: program A (sub-inhibiting treatment on Candida) which dispensed 50 J/cm$^2$ and program B (treatment associated with a moderate cytototoxicity on Candida) which dispenses 100 J/cm$^2$. After 24 h incubation at 37 °C with virus, Candida biofilms were exposed or not to laser beam. Afterwards, washing and scraping for 1′ were performed and the load of infectious virus in the detached biofilm was determined by end-point titration on VERO cells. The same treatment was carried out also in culture wells without C. albicans (controls). The IC$_{90}$ of UVA1 treatment on HSV-1 was determined with or without Candida biofilm. Thus, after 24 h of incubation at 37 °C with virus, Candida biofilms were exposed or not to laser beam. Afterwards, washing and scraping for 1′ were performed and the load of infectious virus in the detached biofilm was determined by end-point titration on VERO cells. 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Afterwards, washing and scraping for 1′ were performed and the load of infectious virus in the detached biofilm was determined by end-point titration on VERO cells.
VERO cells were treated with the same doses of the two antivirals. After 24 h of incubation at 37 °C, plates were frozen and thawed, then the viral titer of each well was determined by end-point titration on VERO cells.

In another set of experiments aimed at determining drug IC$_{50}$, twofold dilutions of each drug were added to wells with infected VERO cells in the presence and in the absence of Candida biofilm. The viral yield in each group was titrated 24 h later by plaque assay. For acyclovir, concentrations ranging from 3 to 50 µM were assayed, while for foscarin from 0.019 to 2.4 mM. A dose–response curve was then elaborated and the IC$_{50}$ was determined for each drug, with or without Candida biofilm.

**Statistical analysis**

The data reported in figures are the mean values (± standard deviation) from at least three different experiments performed. The results were analyzed by the two-tailed Student’s t test and were considered significant when p < 0.05.

**Results**

Laser radiation impact on Candida biofilm

Initially, the effects of laser treatment on biofilm formation or maintenance of mature biofilm were investigated. Accordingly, Candida cells were treated with different doses of UVA1 either just after seeding onto cell culture plates, namely before biofilm formation (pre-treatment), or after 48 h of growth as biofilm (post-treatment). In both cases, the residual metabolic activity and the total biomass were assessed by XTT and CV assay, respectively. The results obtained are shown in Fig. 1 (panel a = pre-treatment, panel b = post-treatment). The OD values of biofilm exposed to laser were similar to those of unexposed cultures for treatments up to a fluency of 60 J/cm$^2$ regardless of the fact that a pre- or a post-treatment had been performed. In contrast, starting from 150 J/cm$^2$ fluency, laser exposure caused a reduction in OD values, with major decrease being observed when using pretreatment (upper panel) irrespective of the assay performed; differently, only metabolic activity (as assessed by XTT) but not total biomass (as assessed by CV assay) were impaired by laser treatment according to the Protocol B. Finally, 100 J/cm$^2$ fluency caused an OD value reduction with a border line significance (p = 0.06) both in pre-treatment and post-treatment (in this case only for XTT values). These results indicate that a low intensity laser radiation (≤ 60 J/cm$^2$) significantly affects neither biofilm formation nor its maintenance.

Candida biofilm impact on laser antiviral activity

On the basis of the results obtained by laser radiation on Candida, two laser conditions were employed to carry out the experiments on antiviral activity of laser treatment: protocol A which dispenses 50 J/cm$^2$ (sub-inhibiting against biofilm) and protocol B which dispenses 100 J/cm$^2$ (the lowest fluency exerting a mild anti-biofilm activity).

In order to evaluate whether virus interaction with Candida biofilm can influence antiviral activity of laser beam, Candida biofilms co-cultured with HSV-1 infected cells for 24 h were exposed or not to laser beam and then the residual load of infectious virus in the biofilm was determined. The results are depicted as viral load reduction in Fig. 2. In control cultures without biofilm, protocol A reduced the amount of HSV-1 by 2 Logs (3.1 TCID$_{50}$ vs 1.1 TCID$_{50}$ for untreated and treated cultures, respectively), while in biofilm cultures the decrease was of 1 Log (3.1 TCID$_{50}$ vs 2.1 TCID$_{50}$ for untreated and treated cultures, respectively). Similar results were obtained applying protocol B: in this case, in the absence of biofilm, virus titer decreased from 3.1 TCID$_{50}$ to 1 TCID$_{50}$ whereas in biofilm cultures the virus titers were 3.1 TCID$_{50}$ vs 1.9 TCID$_{50}$ for untreated and treated cultures, respectively.

The anti-HSV-1 IC$_{90}$ values of laser treatment in the two conditions were ascertained by treating suspensions of HSV-1 in the presence or absence of Candida biofilm with increasing laser fluencies between 10 and 60 J/cm$^2$ and then titrated by plaques assay. The results showed an increased value in the presence of biofilm (18.4 J/cm$^2$ vs 25.6 J/cm$^2$).

Candida biofilm impact on antiviral drug activity

Next, the influence of Candida biofilm on the antiviral activity of acyclovir and foscarin was assessed. To this purpose, HSV-1 infected VERO cells were incubated with or without Candida biofilm in the presence or absence of antiviral drug for 24 h. The residual viral load was evaluated and the results, shown as viral load reduction, are shown in Fig. 3. The virus titers of VERO control cultures (no biofilm) decreased by 2.3 Log (from 3.7 TCID$_{50}$ to 1.4 TCID$_{50}$) when exposed to acyclovir and only by 0.5 Log (from 1.6 TCID$_{50}$ to 1.1 TCID$_{50}$) if the treatment had been performed in the presence of Candida biofilm. Similarly, using foscarin, the virus titers decreased by 1.4 Log in control cultures without biofilm (from 4.5 TCID$_{50}$ to 3.1 TCID$_{50}$) and only by 0.2 Log when the treatment had been performed in the presence of Candida biofilm (from 3.1 TCID$_{50}$ to 2.9 TCID$_{50}$).

In order to establish the IC$_{50}$ values of the 2 drugs, HSV-1 infected VERO cells were treated with increasing doses of the two antivirals in the presence or absence of Candida biofilm. Then residual HSV-1 infectivity was assessed with plaque assay. We found that acyclovir IC$_{50}$ value increased fourfold, being 5.41 and 22.62 µM, in the
absence and in the presence of Candida biofilm, respectively. Similar results were obtained for foscarnet: the IC$_{50}$ value raised from 54 µM in the absence of biofilm to 661 µM in VERO cultures co-incubated with Candida biofilm. In this case, the increase was 12-folds.

Lastly, we evaluated whether the observed reduction in acyclovir and foscarnet efficacy against HSV-1 can be ascribed to fungus growth manner. Thus, HSV-1 infected VERO cells were exposed to C. albicans grown in two different patterns: as a biofilm or planktonic, namely, in the absence or presence of a glass slide in the culture well. By direct microscope observation, we observed that when the Candida strain we used (50vr) [17] was cultured on a cover glass, only planktonic but not sessile Candida was observed.

Co-cultures of Candida and HSV-1 infected cells were exposed to acyclovir and foscarnet for 24 h and the viral loads of the different cultures were then titrated. Figure 4 shows these results, depicted as viral load reduction. In control cultures without Candida, the antiviral treatments caused a reduction of 4 Logs (from 5.8 TCID$_{50}$ to 0.8 TCID$_{50}$) and 3.3 Logs (from 5.8 TCID$_{50}$ to 2.5 TCID$_{50}$) for acyclovir and foscarnet, respectively. In contrast, in cultures with Candida, the virus titers decreased by about 1 Log with either drug, regardless of the fact that the cover glass slide was or was not present: for acyclovir, the virus yield values were 3.3 TCID$_{50}$ with planktonic Candida and 3.5 TCID$_{50}$ with Candida biofilm; for foscarnet, the values were 3.8 TCID$_{50}$ and 3.2 TCID$_{50}$.
Discussion
Biofilm, a major problem in clinical practice, is a structured community usually formed by different microbial types, mainly bacteria and fungi. Few studies are available on the existence of virus entrapping microbial biofilm and they focus mostly on aquatic biofilms [25]. Recently, we demonstrated in vitro that viruses, such as HSV-1 and Coxsackie Virus B5, while embedded in C. albicans biofilm, still retain their infectivity [14]. We also observed that such intra-biofilm localization partially reduces virus particles sensitivity to hypochlorite. By that same in vitro model, here we demonstrated that Candida biofilm protected HSV-1 also from pharmacological treatments (acyclovir and foscarnet, the most used drugs against HSV-1 infections). Moreover, we documented a virucide activity exerted by UVA1 laser and that such activity was decreased in presence of Candida biofilm.

Different hypotheses can be considered to explain such phenomena.

Firstly, a reduced antiviral drug availability might occur on the existence of virus entrapping microbial biofilm and they focus mostly on aquatic biofilms [25]. Recently, we demonstrated in vitro that viruses, such as HSV-1 and Coxsackie Virus B5, while embedded in C. albicans biofilm, still retain their infectivity [14]. We also observed that such intra-biofilm localization partially reduces virus particles sensitivity to hypochlorite. By that same in vitro model, here we demonstrated that Candida biofilm protected HSV-1 also from pharmacological treatments (acyclovir and foscarnet, the most used drugs against HSV-1 infections). Moreover, we documented a virucide activity exerted by UVA1 laser and that such activity was decreased in presence of Candida biofilm.

Different hypotheses can be considered to explain such phenomena.

Firstly, a reduced antiviral drug availability might occur within biofilm, due to either drug molecules engagement by the EPS organic material or aspecific binding to fungal cells surface. Indeed, it is well known that the presence of many organic (proteins) or inorganic (electrolytes, divalent cations) substances leads to decrease or complete inhibition of disinfectant activity, both in vitro and in vivo [26, 27]: for instance, bovine serum albumin is indeed used to this purpose in studies on antimicrobial molecules. Alternatively, extracellular enzymes secreted
by fungal cells in the EPS might directly interact with drug molecules, thus impairing their efficacy. Acyclovir/ foscarnet activity was also tested in the presence of the same Candida strain grown as either planktomic form or biofilm. In both cases, a similar reduction in antiviral activity, with no significant difference, was observed, suggesting that EPS matrix production is not essential to affect drug activity. On these bases, drug-aspecific binding to fungal cells, followed or not by cell entry, could be the most likely hypothesis: the huge amount of biomass presents in both conditions, in one case forming a floating mycelium network and in the other a sessile biofilm structure, might represent a major hindrance to virus-drug interaction. An indirect support to this hypothesis comes from immunofluorescence studies: we observed that HSV-1 antigens or infected cells are more concentrated in areas with higher hyphae density (unpublished data). On the other hand, secretion of enzymes interacting with drug molecules cannot be ruled out: in both growth patterns, Candida excretes a plethora of molecules which could interfere with drug action.

As far as UVA1 irradiation, laser emission is currently used in medicine for topical treatment of different skin and dental diseases as well as for disinfection of surfaces, surgical instruments and water. We observed that when HSV-1 suspensions are exposed to UVA1 light at 355 λ, they undergo a significant inactivation. However, this antiviral activity is significantly affected by the presence of Candida biofilm. In particular, while HSV-1 infectivity was reduced by 2 Logs in the absence of biofilm, both at 50 or 100 J/cm², infectivity was reduced by only 1 Log when biofilm was present. Consequently, IC₉₀ values are 25.6 and 18.4 J/cm² with and without biofilm, respectively. This reduction in drug efficacy could be likely due to energy absorption by the organic mass: although most of the biological molecules have an absorbance peak at low frequencies (210–270 λ), a lower absorbance is still present at higher frequencies (such as 355 λ) and might be sufficient to subtract energy for virus inactivation.

Evidence exists on the mechanisms of laser action on mammalian cells; in particular, UVA1 radiation induces apoptosis by two mechanisms, the one triggered by singlet oxygen species production, the other involving damages to cellular mitochondria [28]. Reactive oxygen species cause damages to fungal cell wall, lipid membranes, nucleic acids, and react with organic molecules in biofilm EPS leading to a partial/complete inactivation of such molecules: bovine albumin represents an example. As said before, it can neutralize the antimicrobial activity of chemical and physical treatments. It is a globular structured protein of high molecular mass, with a single sulfhydryl (SH) group at amino acid residues (Cys-34), which might react with free radicals [29]. Consequently, the large mass of fungal cells with its huge amount of organic target for radiated UVA light as well as the abundant organic material in the EPS might quench laser energy and subtract power, in turn reducing its efficacy on virus particles. Also heat generation by laser irradiation must be considered. As a matter of fact, a photothermolysis effect is among the mechanisms by which UVA irradiation exerts its biological activities [30]: this heating can be responsible, at least partially, for HSV-1 inactivation, considering virus fragility due to the envelope presence. The thick biofilm lime could reduce this heating effect. Finally, the UVA1 laser antimicrobial activity can be ascribed to the production of toxic molecules caused by its interaction with polystyrene of the tissue culture plate. It is well known that exposure to UVA radiation can cause significant degradation of many materials, inducing photooxidative degradation which results in breaking of the polymer chains and free radical production [31]. Again, dispersion in and sequestration by the biofilm mass could reduce the availability for virus of these antimicrobial molecules.

On the whole, our present and previous data [14] show that HSV-1 encompassed in Candida biofilm can retain its infectivity and, more important, is protected from inactivation by chemical (hypochlorite) [14], physical (laser) and pharmacological treatments. Based on these findings, we may envisage that, in vivo, C. albicans biofilm may represent not only a persistent reservoir of fungal cells but also of infectious virus; in fact, circulating virus particles/virus-infected cells, during viremia infections, might be retained and protected within biofilm and, later on, might be released for further dissemination. Accordingly, the presence of Candida biofilm should represent an additional health risk factor, given its ability to entrap/release also viral particles, efficiently protected against conventional antiviral treatments; such a possibility might be dramatic especially in immunocompromised patients. Finally, also the chemo-physical procedures aimed at warranting virus decontamination of materials and surfaces should be carefully reconsidered, since their efficacy might be drastically impaired by the concomitant presence of fungal biofilm.

**Authors’ contributions**

CA acquired a great part of data, he analyzed and interpreted the data regarding all experiments and was the major contributor in writing the manuscript. AS gave important contributions to acquisition and analysis of data regarding laser experiments. SP contributed to experiment preparation. EVT set the in vitro model of biofilm and virus. BP provided the laser machine. EB edited the manuscript. CC gave substantial contributions to conception and design of project and to interpretation of data and supervised manuscript writing. All authors read and approved the final manuscript.

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