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Elastin increases biofilm and extracellular matrix production of *Aspergillus fumigatus*

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ABSTRACT

Aspergillus fumigatus is an opportunistic saprobe fungus that accounts for 90% of cases of pulmonary aspergillosis in immunosuppressed patients and is known for its angiotropism. When it reaches the respiratory tract, *A. fumigatus* interacts with structural components and blood vessels of the lungs, such as elastin. To understand the effect of this structural component, we examined the effect of elastin on the production and development of the biofilm of *A. fumigatus*. In RPMI containing 10 mg/mL of elastin, a significant increase (absorbance $p < 0.0001$; dry weight $p < 0.0001$) in the production of biofilm was observed in comparison to when RPMI was used alone, reaching a maximum growth of 18.8 mg (dry weight) of biofilm in 72 h. In addition, elastin stimulates the production ($p = 0.0042$) of extracellular matrix (ECM) and decreases ($p = 0.005$) the hydrophobicity during the development of the biofilm. These results suggest that elastin plays an important role in the growth of *A. fumigatus* and that it participates in the formation of thick biofilm.

Introduction

Aspergillus fumigatus is an opportunistic saprobe fungus that accounts for 90% of pulmonary aspergillosis cases in immunosuppressed patients. This disease can exhibit various clinical forms, mainly consisting of allergic bronchopulmonary aspergillosis, aspergilloma, and invasive aspergillosis (IA), which are important causes of morbidity and mortality ranging from 70 to 90%.[1,2]

In aspergilloma and IA, *A. fumigatus* behaves as a multicellular community surrounded by an extracellular matrix (ECM), which is characteristic of a biofilm[3,4] and may explain, together with histological evidence, the resistance to antifungal agents when these clinical forms are treated.[5,6]

The development of this fungus within the lungs and the angiotropism[7,8] allow this microorganism to be in direct contact with elastin, one of the main structural components of the

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lungs and blood vessels, which is fundamental for their physiology. Correlation between elastase production by *A. fumigatus* and the development of IA has been observed.[9]

It has recently been demonstrated the influence of host factors such as serum components, as fetuin A,[10] and extracellular DNA[11] in the promotion of growth of *A. fumigatus* biofilm; however, no studies have investigated the influence of lung tissue constituents on the promotion of biofilm development.

In this perspective, the aim of this work was to determine the influence of elastin in the growth and development of the biofilm of *A. fumigatus*.

Materials and methods

Fungal strain and growth conditions

Based on data obtained from previous analysis of virulence factors such as biofilm and gliotoxin production, and ability to cause pulmonary aspergillosis in mice (unpublished data), we selected two isolates of *A. fumigatus*, URM5992 (environmental origin) and URM6575 (clinical sample), from the Culture Collection University Recife Mycology (URM) of the Federal University of Pernambuco (Universidade Federal de Pernambuco – UFPE), Recife, Pernambuco (PE), Brazil, were used. The isolates were maintained at 28 °C in malt extract agar.

Growth conditions and inoculum standardization

A. fumigatus isolates were grown on Sabouraud dextrose agar at 37 °C for 72 h. The conidia were collected by washing the surface of the culture with 5 mL of phosphate buffer saline (PBS), pH 7.2, supplemented with 0.025% (v/v) Tween 20. The inoculum was adjusted to 1×10^5 cells in RPMI 1640 (Sigma-Aldrich Corporation, USA) and buffered to pH 7.0 with 0.165 M MOPS (Sigma-Aldrich Corporation, USA) for the production of biofilm in 96-well plates.[12] For quantification of the dry weight, another inoculum was adjusted to 3.75×10^4 cells/cm^2.[10]

Production of A. fumigatus biofilm

A. fumigatus biofilm were produced in flat-bottom 96-well polystyrene plates. Then, 200 μL of the standardized cell suspension of each *A. fumigatus* isolate was added separately in MOPS-RPMI 1640 (Sigma-Aldrich Corporation, USA) or MOPS-RPMI 1640 containing elastin (RPMI/Elastin) (Sigma-Aldrich Corporation, USA) at concentration of 10 mg/mL for each time (24, 48, and 72 h). Plates were incubated at 37 °C. For each time interval, the culture medium was removed from the wells, and the cells were washed three times with PBS, pH 7.2, to remove all non-adherent cells.[12]

To quantify the dry weight of the biofilm, 3 mL suspensions of each isolate were placed separately in 6-well polystyrene plates with MOPS-RPMI 1640 or RPMI/Elastin (10 mg/mL), incubation times, and temperatures listed above.[10]

Biofilm quantification

Biofilm was quantified using the technique developed by O'Toole and Kolter[13] and subsequently modified by Mowat et al.[12] The plates were dried, and 100 μL of 0.5% (w/v) crystal violet solution was added for 5 min. The solution was removed by thorough washing under running water. Biofilms were unstained by adding 100 μL of 95% ethanol to each well for 1 min. The ethanol was transferred to another microtiter plate (96-well), and the absorbance was measured at 570 nm (A570) using a VarioskanFlash fluorescence meter with SkanIt™ 2.4.5 RE software (Thermo Fisher Scientific, USA).

Quantification of the biofilm biomass (dry weight)

After the predetermined time, the biofilm was removed by scraping and filtered through paper filters (Miracloth/22 μm, Merck, Germany), which were then dried to a constant weight.[10]

Quantification of the ECM

The biofilm formed in RPMI and RPMI/Elastin (10 mg/mL) for 48 h at 37 °C were stained by the addition of 100 μL of a solution of 25 μg/mL Alexa Fluor 488 (CAAF; Life Technologies, Germany) in PBS, followed by incubation for 45 min at 37 °C and stirring at 250 rpm. The biofilm was washed three times with PBS.[11] The fluorescence intensity was measured using a VarioskanFlash fluorescence meter with SkanIt™ 2.4.5 RE software (Thermo Fisher Scientific, USA) at excitation and emission wavelengths of 485 nm and 520 nm, respectively. CAAF stock solutions of 5 mg/mL were stored at −20 °C and thawed immediately before use.

Quantification of biofilm hydrophobicity

A microsphere adhesion assay with fluorescent orange sulfate-modified latex microspheres (0.806 μm, Sigma-Aldrich Corporation, USA) was used to test biofilm hydrophobicity. The biofilm in RPMI alone and RPMI/Elastin (10 mg/mL) were washed with 0.1 M KNO_3, pH 6.5, and then mixed with an equal volume of the microsphere solution (10^9/mL). Subsequently, the mixture was stirred for 30 s and extensively washed with the same solution.[3] The amount of fluorescence emitted resulting from the adherence of the microspheres to the hyphae was measured with a VarioskanFlash fluorescence meter with SkanIt™ 2.4.5 RE software (Thermo Fisher Scientific, USA) at excitation and emission wavelengths of 520 nm and 540 nm, respectively.

Biofilm microscopy

For microscopic analysis, the biofilm was grown on coverslips (22 mm × 22 mm) in RPMI and RPMI/Elastin (10 mg/mL) at 37 °C for 48 h in 6-well polystyrene plates. The coverslips were removed, and the biofilm was analyzed.

To visualize the structure of the biofilm, 100 μL of Calcofluor White® (Sigma-Aldrich Corporation, USA) and excitation/emission filters of 346/433 nm were used to obtain a blue color. The ECM quantification and hydrophobicity assays were conducted as described above for the quantification of fluorescence.

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