Original Article

Cytotoxicity of *Aspergillus fumigatus* Culture Filtrate Against Macrophages

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Abstract

Aspergillus fumigatus causes serious, life-threatening human infection, and is one of the most important pathogenic fungi. Little is known, however, about its mechanism of infection or its virulence factors.

To learn about its virulence factors, the effect of the culture filtrate of *A. fumigatus* on macrophages was studied. When cocultured with *A. fumigatus* in 96-well microplates, murine peritoneal macrophages showed significant morphological changes indicating serious cellular damage, even when the macrophages were not in direct contact with the fungus. Then culture filtrates of *Aspergillus* spp., *A. fumigatus*, *A. flavus*, *A. terreus* and *A. niger*, were prepared by culturing the fungus in 96-well or 24-well microplates for 24 h, and the effect of the culture filtrates was determined by culturing macrophages with or without culture filtrate. When cultured with the culture filtrate of *A. fumigatus* at a concentration of 1 % or higher, macrophages demonstrated significant morphological changes, leading to their death. Treatment with heat greatly lowered the activity of the culture filtrate. In contrast, culture filtrates of *A. terreus* and *A. flavus* showed no detectable effect on macrophages, whereas *A. niger* did display a similar, but much weaker effect.

Our study strongly suggests that *A. fumigatus* releases a toxic product (s) in the medium very rapidly, and this may be critically involved as the virulence factor in human infection, at least in part, by causing serious injury to macrophages.

Key words: *Aspergillus fumigatus*, macrophage, culture filtrate, virulence factor

Introduction

Aspergillosis is one of the most important systemic mycoses in Japan, and it has been on the increase in recent years. The infection primarily involves the lung, followed by the brain and other internal organs, and is often life-threatening. The most common and virulent etiologic agent causing systemic aspergillosis is *Aspergillus fumigatus*. The mechanism of the pathogenicity of this fungus has been variously investigated, and many virulence factors were proposed, including protease¹⁾, Asp-hemolysin²⁾, mitogillin^{3, 4)}, fumagillin, helvolic acid, rodlet⁵⁾ and gliotoxin^{6–9)}. However, none of them has been confirmed to play a key role

in the pathogenicity, and thus the virulence mechanism is yet to be identified.

Leukocytes, such as neutrophils and macrophages, are known to play a major role in the defence of fungal infection. While studying the antifungal activity of macrophages, we found that they were readily destroyed when cocultured with *A. fumigatus* even when they were not in direct contact with the fungus (unpublished observation). On the basis of this finding, we suspect that *A. fumigatus* may elaborate and release some toxic substance (s) which impairs the antifungal activity of macrophages, and that this process could be an important virulence factor of this facultative fungus.

Although various aspects of the culture filtrate of *A. fumigatus* have been studied, no report has ever suggested its serious effect on macrophages. The purpose of this study was to determine

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what effect there was on macrophages, and, if any, also to ascertain whether the same phenomenon as that seen in the coculture system takes place.

Materials and Methods

Fungi

Four isolates of *A. fumigatus* (IFM 41362, IFM 47439, IFM 47450, IFM 49824) were used throughout the study. IFM 41362 and IFM 47439 were from patients with invasive pulmonary aspergillosis, and IFM 47450 and IFM 49824 from patients with allergic bronchopulmonary aspergillosis (ABPA). Other isolates used were from: *A. niger* (IFM 47753 and IFM 49437 were isolated from aspergillosis patients, and IFM 47800, a type strain, was isolated from the environment), *A. flavus* (IFM 47127 was isolated from an empyema patient; IFM 47130 was isolated from an aspergillosis patient, and IFM 50915 from an ABPA patient) and *A. terreus* (IFM 47047 was isolated from a patient of otomycosis, IFM 47277 and 49623 were isolated from ABPA patients). All of the isolates were stored and maintained in the culture collection of our laboratory.

Each isolate was cultured on potato dextrose agar (Difco Laboratories, Detroit, Michigan, USA) plates at 35°C for 7 days for sporulation. The spores were collected with phosphate-buffered saline (PBS) with 0.05% Tween 20 and washed. The spore suspension was adjusted to 5×10^4 /ml in RPMI 1640 (Sigma, St. Louis, Missouri, USA).

Macrophages

Murine peritoneal macrophages were collected as described by Levitz and DiBenedetto¹⁰⁾. In brief, eight-week-old male BALB/c mice (Charles River Japan Inc., Yokohama, Japan) were anesthetized with ether and venous blood was collected. The peritoneal cavity was then rinsed with RPMI 1640 and peritoneal cells were collected, rinsed and their concentration was adjusted to 2×10^5 /ml with RPMI 1640. One hundred microliters of the suspension put into each well of a 96-well microplate (Nalge Nunc International, Roskilde, Denmark). The viability of nucleated cells as measured by Eosin Y dye exclusion was >87%. Macrophage content in the cell suspension was more than 70% as estimated by differential counts of stained cells (Dif Quick Stain, Baxter Healthcare Corporation, McGaw Park, IL, USA), and peritoneal cells were used as resident peritoneal macrophages thereafter. In some experiments, peritoneal cells were cultured in a CO₂ incubator at 37°C for 2 h, non-adherent cells were removed, and the adherent cells were

used.

Aspergillus fumigatus culture filtrate

To obtain culture filtrates, 96-well and 24-well flat-bottomed microplates (Sumitomo Bakelite Co., Ltd., Tokyo, Japan) were used. Spore suspensions of *A. fumigatus* were put into each well (100 μ l for 96-well microplates and 400 μ l for 24-well microplates, respectively), and were cultured in a CO₂ incubator at 37°C. The depth of the suspension in the wells was approximately 2–3 mm under both conditions. Twenty-four hours later, the culture medium was collected, sterilized with a filter with a pore size of 0.22 μ m (Amicon Inc., Beverly, MA, USA), and stored at –80°C until use. The pH of some samples was determined using a pH meter (Yanagimoto Inc., Japan). In some experiments, the culture filtrate was heat-treated (100°C for 10 min) before adding to the macrophages.

Morphological changes of macrophages cocultured with *Aspergillus* spp.

Macrophages (5×10^4 /ml) were put into wells of a 96-well microplate with 10% of autologous serum. Spores of *Aspergillus* spp. were diluted, then added to the wells to achieve an E/T ratio of 100/1. The cells were then cultured at 37°C for 48 h, and were checked periodically under a microscope for morphological changes.

Morphological changes of macrophages treated with culture filtrate

One hundred microliters of macrophage suspension was put into wells of a 96-well microplate with 10% of autologous serum, and the culture filtrate was added at concentrations of from 1 to 10%. The cells were incubated at 37°C in a CO₂ incubator, and the macrophage morphology was checked frequently with an inverted microscope for up to 24 h. The viability of the macrophages was also determined by Eosin Y staining. Macrophages without culture filtrate were used as control. To determine the effect on the viability of macrophages, 50 μ l of macrophage suspension with or without the culture filtrate was placed on sterile slides and incubated in a humidified CO₂ incubator at 37°C for 24 h. At 1 h, 4 h, 12 h and 24 h, 50 μ l of Eosin Y solution (0.2%) was added to the cells and the viability was determined.

Results

Changes in macrophages cocultured with *Aspergillus* spp. (Fig. 1)

To determine the effect of the presence of

Fig. 1. Morphological changes of murine peritoneal macrophages when cocultured with *A. fumigatus*. Macrophages were cocultured with spores of *A. fumigatus* for 24 h (A). Note that macrophages were rounded off and apparently seriously damaged even without direct contact with the fungi. In contrast, macrophages grew in a spindle-shaped manner when cultured alone in RPMI 1640 (B).

Aspergillus spp. on macrophages, murine peritoneal macrophages were cocultured with spores of *Aspergillus* spp. at 37°C for 24 hours. Some macrophages were cultured alone for control. When checked by microscope after 24 hours, macrophages used for control were seen to be growing in a spindle-shaped manner (Fig. 1B). All of the *Aspergillus* isolates grew in a mycelial form when cultured alone. *A. fumigatus* also changed into mycelial form when cocultured with macrophages; however, the macrophages did not grow in a spindle shape, but rather in a small round form (Fig. 1A). Of particular interest was the fact that these changes were also evident in macrophages that had no direct contact with mycelia. In contrast, macrophages remained morphologically intact when cocultured with the other *Aspergillus* spp. used.

Effect of culture filtrate of *A. fumigatus* on macrophages (Fig. 2)

When the culture filtrate of *A. fumigatus* was

Fig. 2. Morphological changes of murine peritoneal macrophages when treated with 10% of *A. fumigatus* culture filtrate for 24 h. Macrophages show similar changes as seen in the coculture system with hyphae of *A. fumigatus*. Macrophages have become rounded and some of them released granules (A). Those cultured without culture filtrate served as control (B).

added to macrophages, similar changes were seen as in the coculture system; after 24 hours, macrophages had rounded off and shrunk in size; some of them showed intracellular granules, and sometimes granules were released extracellularly. In contrast, control macrophages grew well in a spindle-shaped manner, and no granule formation was seen. These findings began to become evident from 1.5 h after start of the treatment with the culture filtrate, and the cellular changes rapidly became more overt thereafter. In terms of the concentration of culture filtrate, the changes were seen even when only 1% of culture filtrate was added, and they became more severe as the concentration was increased. The pH of the culture filtrate was found to be around 7.4. When the macrophages were exposed to heat-treated culture filtrate, these findings were not detectable at 1 or 2% of the filtrate, but became detectable at 5%. The viability of macrophages checked with Eosin Y staining decreased with the duration of treatment: 72%, 47%, 45%, and 2% at 2 h, 4 h, 6 h, and 24 h, respectively. After 6 h, the number of macrophages apparently decreased and

numerous cellular fragments appeared in the medium, suggesting that many macrophages had been fragmented and destroyed. The culture filtrates taken from 96-well and 24-well microplates revealed exactly the same results.

Effect of culture filtrate of other *Aspergillus* spp. on macrophages

When 1 % of culture filtrate of *A. terreus* or *A. flavus* was added to the culture medium of macrophages, no effect was seen on macrophage morphology. Even when the concentration was increased to 20%, there was no difference between culture filtrate-treated and non-treated macrophages. In *A. niger*, morphological changes were not seen at 10%, but at 20% of culture filtrate slight changes were observed. Although one of the three isolates had come from the environment, there was no clear difference between it and the clinical isolates.

Discussion

Our study disclosed that 1) the culture filtrate of *A. fumigatus* has a toxic effect on macrophages, 2) the cytotoxic action is rapid and potent, causing morphological changes and even death in macrophages, 3) the activity of the culture filtrate becomes overt within 24 h of culture, 4) this activity is greatly decreased when heated at 100°C for 10 min, and 5) the culture filtrate of other *Aspergillus* species (*A. niger*, *A. flavus*, *A. terreus*) has very weak or no effect on macrophages. From these results it is strongly suggested that *A. fumigatus* elaborates some macrophage-toxic substance (s) in the culture filtrate.

Studies have been reported the culture filtrate of *A. fumigatus* on mammalian cells⁶⁻⁹⁾. Although investigations of the effect on phagocytes have also been carried out on macrophages and polymorphonuclear leukocytes^{8,11)}, the previous findings were limited to the suppression of the function of phagocytes such as phagocytosis, chemotaxis, and O₂⁻ release. The morphological changes and destruction of macrophages seen in our study have not been reported and, to our knowledge, this is the first report of its kind.

The characteristics of culture filtrate depend on the culture conditions, namely the medium, temperature, concentration of fungi, duration of culture, type of container, etc. In this regard, our system for collecting culture filtrates had two specific features, the duration and the container for the incubation. In terms of incubation time, most of the previous studies of *A. fumigatus* culture filtrate required 3-7 days of culture to achieve detectable levels of activity^{8, 11, 12)}. It may

be rather unreasonable, however, to assume that toxicity which starts to be exerted after several days of culture plays a critical role in the actual infection. Therefore, the rapid onset of the activity is another distinction of our culture filtrate, and suggests that this activity may have a crucial function in the actual infection.

The other feature of our culture system concerns the container. In earlier studies the culture filtrate of *A. fumigatus* was generally made in large flasks without shaking^{8,12)}, which rendered the environment of the fungi to be different from the actual one *in vivo*. For instance, fungi growing at the bottom of 300 ml of culture medium in a 500 ml flask are presumably exposed to a completely different environment from the actual *in vivo* condition, particularly from the viewpoint of aeration compared with that in the infected lung. In contrast, the depth of the medium used in our study was only 2~3 mm, and aeration of the medium was believed to be quite efficient due to its thin layer. Although the reason for the rapid production of the toxic substance in our study is not fully understood, we suspect that the efficient aeration might have had a critical impact on the metabolism of the fungi, accelerating the production of the toxic substance (s) that remained undetectable in the regular culture system. This high level of aeration provided our culture system with another advantage, namely that the culture environment was much closer to that of the actual infection because the fungi were exposed to a high content of oxygen in the lung, the primary target organ of aspergillosis.

The active substance (s) in our culture filtrate is unknown. Among various virulence factors of *A. fumigatus* like protease¹⁾, rodlet⁵⁾, Asp-hemolysin²⁾, restrictocin⁴⁾ and gliotoxin^{8,11)}, gliotoxin may be a possible substance because it shares the similar characters of antiphagocytic activity and inactivation of the activity by heating. However, all of the studies on gliotoxin production to date have claimed that at least three days of culture are required before it exerts these effects in culture filtrate⁸⁾, and the possible relation between gliotoxin and the active substance in our culture filtrate remains obscure. Analysis of these substances with regard to their structure and biological activities is now underway.

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References

- 1) Tang C, Cohen J, Krausz T, Van Noorden S, Holden D: The alkaline protease of *Aspergillus fumigatus* is not a virulence determinant in two murine models of invasive pulmonary aspergillosis. *Infect Immun* **61**: 1650-1656, 1993.
- 2) Ebina K, Yokota K, Sakaguchi O: Studies on toxin of *Aspergillus fumigatus* XVI. Biological properties of Asp-hemolysin as a parasite factor. *Jpn J Med Mycol* **24**: 245-252, 1983.
- 3) Arruda L, Mann B, Chapman M: Selective expression of a major allergen and cytotoxin, Asp fl, in *Aspergillus fumigatus*. Implications for the immunopathogenesis of *Aspergillus*-related diseases. *J Immunol* **149**: 3354-3359, 1992.
- 4) Smith J, Tang C, Van Noorden S, Holden D: Virulence of *Aspergillus fumigatus* double mutants lacking restriction and an alkaline protease in a low-dose model of invasive pulmonary aspergillosis. *Infect Immun* **62**: 5247-5254, 1994.
- 5) Shibuya K, Takaoka M, Uchida K, Wakayama M, Yamaguchi H, Takahashi K, Paris S, Latge J, Naoe S: Histopathology of experimental invasive aspergillosis in rats: pathological comparison of pulmonary lesions induced by specific virulent factor deficient mutants. *Microb Pathog* **27**: 123, 1999.
- 6) Amitani R, Murayama T, Nawada R: *Aspergillus* culture filtrates and sputum sols from patients with pulmonary aspergillosis cause damage to human respiratory ciliated epithelium *in vitro*. *Eur Respir J* **8**: 1681-1687, 1995.
- 7) Hobson R: The effects of diffusates from the spores of *Aspergillus fumigatus* and *A. terreus* on human neutrophils, *Naegleria gruberi*, and *Acanthamoeba castellanii*. *Med Mycol* **38**: 133, 2000.
- 8) Mullbacher A, Waring P, Eichner R: Identification of an agent in cultures of *Aspergillus fumigatus* displaying anti-phagocytic and immunomodulating activity *in vitro*. *J Gen Microbiol* **131**: 1251-1258, 1985.
- 9) Robertson M, Seaton A, Raeburn J, Milne L: Inhibition of phagocyte migration and spreading by spore diffusates of *Aspergillus fumigatus*. *J Med Vet Mycol* **25**: 389-396, 1987.
- 10) Levitz S, DiBenedetto D: Differential stimulation of murine resident peritoneal cells by selectively opsonized encapsulated and acapsular *Cryptococcus neoformans*. *Infect Immun* **56**: 2544-2554, 1988.
- 11) Murayama T, Amitani R, Ikegami Y, Nawada R, Lee W, Kuze F: Suppressing effects of *Aspergillus fumigatus* culture filtrates on human alveolar macrophages and polymorphonuclear leucocytes. *Eur Respir J* **9**: 293-300, 1996.
- 12) Speth C, Rambach G, Lass-Floerl C, Wurzner R, Gasque P, Mohsenipour I, Dietrich M: Culture supernatants of patient-derived *Aspergillus* isolates have toxic and lytic activity towards neurons and glia cells. *FEMS Immunol Med Microbiol* **29**: 303-313, 2000.