Aspergillus mycotoxins and their effect on the host

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Aspergillus fumigatus is known to produce various immunosuppressive mycotoxins including gliotoxin. However, none of these mycotoxins has been confirmed as being directly related to the pathogenesis of aspergilli. Recent studies have made substantial progress in the determination of mycotoxins as virulence factors. Gliotoxin was found to be produced much faster than previously believed under certain culture conditions, such as at 37°C and under high oxygen content, which is close to the environment in the host. Gliotoxin was also found to be detectable in the sera of aspergillosis mice and of aspergillosis patients. Based on these findings, it is becoming evident that gliotoxin is produced in the infected organs of patients of aspergillosis at a significant level. In addition to these known mycotoxins, A. fumigatus produces many mycotoxins apparently different from known toxins. From the aspect of gene analysis, the deletion of laeA was found to block the expression of metabolic gene clusters such as sterigmatocystin, and the gene is also expected to be related to the production of gliotoxin. The significance of mycotoxins as virulence factors will hopefully be clarified in the near future.

Keywords Aspergillus, mycotoxin, gliotoxin, virulence factor

Introduction

Mycotoxins, which are produced by many species of fungi, are secondary metabolites that are toxic to humans and animals. Most of them are of small molecular sizes (MW < 300) [1]. Various investigations of mycotoxins have been carried out over the years, most from the viewpoint of food intoxication and its prevention [2]. Mycotoxin food contamination causing mycotoxicoses in animals and humans has been a serious problem, and remains so in some developing countries. Pathogenic Aspergillus spp. also produce mycotoxins. These toxins are known for their strong and varied biological activities. For example, aflatoxin, the most well-known and well-investigated mycotoxin, is known to carry the most potent carcinogenic activity as a natural product. It also carries acute toxicity to various human cells such as hepatocytes, renal cells, lung epithelioid cells, etc., as well as various immunosuppressive activities [3–5]. Many other mycotoxins have fairly similar activities [6].

Considering the potent biological activity, it is likely that mycotoxins work as virulence factors in the development of aspergillosis. However, studies focusing on the relation between mycotoxins and its pathogenesis have been limited, and significance of mycotoxins in the virulence of Aspergillus fumigatus has not yet been demonstrated [7].

Function of mycotoxins and their production by Aspergillus spp. *in vitro*

Until recently, the relationship between mycotoxins and the pathogenicity of the fungi that produce them has received little attention. However, there are many mycotoxins with the capability to alter the defense system of the host, and by this immunosuppressive activity these mycotoxins may help the fungus to invade the host tissue by working as virulence factors. Among mycotoxins produced by Aspergillus spp., for example, Aspergillus flavus produces aflatoxin that suppresses the function of macrophages [8], and Aspergillus ochraceus produces ochratoxin that is known to be cytotoxic to lymphocytes [8], and it suppresses many functions of lymphocytes, monocytes, and granulocytes [9,10].
Although *A. fumigatus* is not known to produce aflatoxin, the fungus is known to produce various immunosuppressive mycotoxins including gliotoxin (MW 326 Da), fumagillin (459 Da), helvolic acid (fumigacin) (569 Da), fumitremorgin A and Asp-hemolysin (16 kDa) [11–13]. Gliotoxin is an alkaloid with a low molecular size which has been known to possess a number of immunosuppressive activities, such as inhibition of superoxide release, migration, microbicidal activity [11,14–17] cytokine release [18] by leukocytes, and T-lymphocyte-mediated cytotoxicity [19]. It is genotoxic [20] and also causes apoptosis in macrophages [21]. Fumagillin is a cyclohexane derivative, and is known as an inhibitor of endothelial cell proliferation and angiogenesis. Asp-hemolysin is a hemolytic toxin and is cytotoxic to neutrophils and macrophages. All of these mycotoxins, i.e., gliotoxin, fumagillin, helvolic acid, and Asp-hemolysin, inhibit the function of leukocytes in terms of migration, superoxide production and fungicidal activity, with the activities of gliotoxin being much stronger than those of the others. However, none of these mycotoxins, including gliotoxin, has been confirmed as being directly related to the pathogenesis of aspergillosis despite their strong activities.

As a general characteristic, mycotoxins are thought to be produced slowly, reaching detectable levels after a long culture period, which means that their release is time-dependent in culture. This slow production means that mycotoxins are unlikely virulence factors. In fact, in order to collect fungal mycotoxins in culture filtrates, fungi were traditionally cultured in conventional poorly-aerated conditions at low temperature (e.g., room temperature), and the mycotoxins were extracted from the filtrates. Under this environment, some days of culture were essential for the production of mycotoxins.

However, the metabolism of fungi depends on the environment, and therefore the culture condition plays a critical role in the production of mycotoxins [22]. Recent studies have found that gliotoxin was produced much faster (from 29 hours after the beginning of incubation) than previously believed under a certain culture condition such as at 37°C [23].

Aeration during the culture was found to be another important factor for the rapid production of gliotoxin. When the culture filtrate of *A. fumigatus* is made in a well-aerated culture system in a medium such as RPMI 1640 at 37°C, the filtrate rapidly (within 15 hours of culture) exhibits potent cytotoxic activity against leukocytes such as macrophages and polymorphonuclear leukocytes at a low concentration (1%). The leukocytes demonstrated significant morphological changes, and were destroyed by exposure to the culture filtrate. These findings were confirmed by examining more than 10 clinical and environmental isolates. In contrast, this activity was not seen or was much weaker in the filtrates of other *Aspergillus* spp. such as *A. flavus*, *A. terreus* or *A. niger*, or when *A. fumigatus* was cultured under poorly-aerated conditions [24]. At lower concentrations, the filtrate of *A. fumigatus* inhibited the migration, phagocytosis and superoxide generation by human polymorphonuclear leukocytes. When injected into murine peritoneal cavities with spores of *A. fumigatus*, the culture filtrate dramatically promoted the development of aspergillosis and shortened the survival of mice [25].

Analysis of the filtrate by gas chromatography-mass spectrometry disclosed that a significant amount of gliotoxin became detectable in the culture filtrate when the cytotoxic activity became evident. When the culture filtrate was made in a special container with direct control of oxygen concentration in the environment, gliotoxin production was found to be dependent on the oxygen concentration of the environment at <14% oxygen in a concentration-dependent manner [26,27]. The primary target of infection by *A. fumigatus* is the lung, the most well-aerated organ. In this sense, the high concentration of oxygen in the lung provides an optimal condition for the production of gliotoxin by *A. fumigatus*.

Furthermore, analysis of the filtrate also disclosed some unknown cytotoxic components other than gliotoxin that were not listed in the library search program, meaning that these components were apparently novel. The active substance was heat labile, soluble in chloroform, and had a small molecular size (<3 kDa). When the cytotoxic activity of the filtrate was compared with authentic gliotoxin, the concentration of gliotoxin (ca. 4 mcg/ml) was found to be too low to be solely responsible for the cytotoxic activity, and it is assumed that the novel substances play a significant additional role in the activity of the filtrate [25].

In addition to the novel active substance described above, there are many studies reporting that *A. fumigatus* produces many mycotoxins apparently different from known toxins [28–31]. The combination effect of these mycotoxins may produce unexpected synergistic results [32].

**Aspergillus mycotoxins in vivo: production and function**

To work as virulence factors, mycotoxins should be produced and be active *in vivo*. From this aspect, studies have been even more limited. In terms of
mycotoxins of large molecular size, ribonucleotoxin (molecular size: 18 kDa) was found in the urine of aspergillosis patients [33,34]. Restrictocin is produced by A. fumigatus and also by non-pathogenic species such as Aspergillus restrictus. It blocks protein synthesis and exerts toxicity to human cells, and was once regarded as a candidate of a virulence factor of A. fumigatus [33]. However, studies using restrictocin-deficient A. fumigatus isolates by gene disruption showed disappointing results, demonstrating no difference in virulence between these strains and indicating that restrictocin is not an important determinant of virulence [35,36].

As for the smaller mycotoxins such as gliotoxin, research had been hampered by a number of technical problems until the recent development of more advanced techniques. Producing antibodies to these non-proteinous small mycotoxins such as gliotoxin had sometimes been problematic owing to their structure, and few reports were available. Recently, Fox et al. [37] reported a new simple method using thyroglobulin-based immunogens, and successfully produced antibodies to gliotoxin and helvolic acid. These antibodies are expected to work as reliable tools for detecting the presence of mycotoxins in infected organs [37].

In terms of the detection of gliotoxin in vivo, only a few cases of gliotoxin being detected in infected tissues have been reported. Bauer et al. [38] reported the presence of gliotoxin in a cow’s udder. Richard et al. [39] made an animal model using turkeys and found a significant amount of gliotoxin in the poults of infected animals. Recently, Reeves et al. [40] detected gliotoxin in the bodies of larvae of experimentally-infected Galleria mellonella, and compared the gliotoxin content between isolates [40]. These findings indicate that A. fumigatus is capable of producing gliotoxin in vivo in some animals. In addition, Lewis et al. [41], using LC-tandem mass spectroscopy, recently showed that a detectable level of gliotoxin was present in the sera of aspergillosis mice. Gliotoxin was also found in the sera of aspergillosis patients [41]. This shows that detection of gliotoxin may be used as a diagnostic tool. It also shows that a significant amount of gliotoxin is present in infected tissues, and a substantial biological effect should be expected. From these findings, it is becoming increasingly evident that gliotoxin is produced at a significant level in the infected organs of patients with aspergillosis. Its role in the pathogenesis of aspergillosis remains to be clarified until a gliotoxin negative strain of A. fumigatus is produced.

The function of mycotoxin in vivo is of primary importance for understanding the pathogenicity of mycotoxins. Some studies have demonstrated the worsening of aspergillosis by giving gliotoxin to infected animals [25,42]. The gene manipulation technique is believed to be a powerful tool for research, and it may help to clarify the function of mycotoxins. However, most mycotoxins are not proteins, and therefore genetic analysis by manipulating genes that directly code the synthesis of the toxins is difficult to perform. Many trials have been undertaken to produce a gliotoxin-deficient strain by this technique, but they were usually unsuccessful. Recently, analysis of genes of Aspergillus spp. related to the production of some secondary metabolites focused on isolated genes encoding the enzymes related to the biosynthesis of aflatoxin and sterigmatocystin. Their expression was also found to be mostly regulated by a product of the regulatory gene aflR. The deletion of laeA (delta laeA) was found to block the expression of metabolic gene clusters, including sterigmatocystin (carcinogen), penicillin (antibiotic), and lovastatin (anti hypercholesterolemic agent) [43,44]. It is expected that the gene might also be related to the production of gliotoxin. However, the gene is unlikely to be specifically related to the synthesis of gliotoxin, and the metabolism of the fungus may result in a change in the synthesis of many metabolites including gliotoxin. Nonetheless, the analysis of this cluster is expected to help our understanding of the role of gliotoxin in the development of aspergillosis. Gene disruption that focuses on the genes more specific to gliotoxin synthesis is warranted.

The future of mycotoxin studies

Recent studies have made substantial progress in the determination of mycotoxins as virulence factors. Although the pathogenicity of aspergillosis may be multifactorial [45], mycotoxins should be examined not just as a source of food contamination but also as possible virulence factors. It is obvious that much remains to be done in the investigation of mycotoxins and their relation to virulence. Recently, treatment by amphotericin B was found to augment the release of gliotoxin by A. fumigatus, possibly causing immunological status deterioration of the host [46]. As the role of mycotoxins in aspergillosis becomes clear, drugs that can control the production of mycotoxins may be a new and attractive target of development.

References


