Investigation on Biofilm Formation by *Aspergillus* spp.

- Effect of Serum Components and Its Relation to Antifungal Susceptibilities

(*Aspergillus* 属による biofilm 形成の研究
- 血清成分の効果と抗真菌薬感受性への影響について)
Aspergillosis is one of the most important deep-seated mycoses in the world, and Japan is not an exception. The disease is caused by fungi of the genus *Aspergillus* comprises a wide variety of disease forms. The most common forms are allergic chronic pulmonary aspergillosis including aspergilloma, and invasive aspergillosis, and bronchopulmonary aspergillosis. Aspergillosis develops mainly in individuals who are defective in the defense system, either from disease or from immunosuppressive drugs, and is a leading cause of death in patients with acute leukemia and hematopoietic stem cell transplantation.

There are a number of species among aspergilli, and *Aspergillus fumigatus* has been reported to be the one which causes aspergillosis most often in patients with leukemia or who have undergone organ/bone marrow transplantation. However, recent studies showed an increase emergence of aspergillosis cases caused by non-*fumigatus* *Aspergillus* species inherently more resistant to antifungal agents in both bone marrow and solid organ transplant recipients.

Most microorganisms preferably grow in a complex milieu, in the form of structured polymicrobial biofilms on both natural and inanimate surfaces. Microorganisms growing together in composite biofilms produce an extracellular matrix (ECM) that binds them to one another and to the in vitro or in vivo substrate. These biofilms are well protected from hostile environments of the host immune system and also resist antimicrobial killing. The transition of a planktonic form of a microbe to a biofilm or a sessile structure (surface-attached) is the result of interactions between different
pathogen and host factors. The formation of these microbial biofilms is a prerequisite event towards the development of invasive disease and it has been reported that these biofilms are involved in about 80% of non-acute infections in humans. However, only recently have studies been initiated to investigate the complicated biofilm phenotype during diseases, especially fungal diseases. Among studies on fungal biofilms, biofilms formed by *Candida albicans* on catheter material and human cell surfaces are the most well-studied fungal systems. In contrast the formation of biofilms by another opportunistic fungus, *A. fumigatus* has just begun to be understood. Although the biofilm is believed to play a pivotal role in the infection, the major players and molecular mechanisms underlying biofilm formation in *A. fumigatus*-mediated diseases remain largely elusive.

Previously we have disclosed that serum, as well as serum glycoprotein fetuin A, promotes both hyphal growth and biofilm formation of *A. fumigatus*. However, the detail of biofilm formation by *Aspergillus* spp., particularly by non-*fumigatus* *Aspergillus* spp. and the effect of serum on these fungi, have been left unattended thus far.

In my research presented here the biofilm-forming capacity of five representative pathogenic *Aspergillus* spp., i.e., *A. fumigatus*, *A. flavus*, *A. nidulans*, *A. niger* and *A. terreus*, was evaluated, and the effect of serum/serum proteins, such as fetal bovine serum (FBS), fetuin A and bovine serum albumin (BSA), on hyphal growth, hyphal branching and extracellular matrix (ECM) formation.

In addition, the antifungal susceptibilities of 171 *A. fumigatus* isolates, which were collected
from all over the country and stored as *A. fumigatus* at the Medical Mycology Research Center, Chiba University during a recent 22-year period, was determined, when they were not forming biofilms and were compared with those forming biofilms. The activity of various antifungal agents in combination was also examined to find an optimal method for the treatment of the intractable fungal disease with biofilms.

Fungal growth was promoted by all serum/serum proteins in all species examined, with the promotion most evident with FBS, followed by fetuin A and then BSA. The effect was most striking in *A. fumigatus* and least in *A. terreus*. An analysis with electronmicroscopy showed a thick ECM layer surrounding the cell wall when treated with FBS particularly in *A. fumigatus*. Hyphal branching was promoted by fetuin A, which was most evident in *A. fumigatus* and *A. nidulans*. When antifungal susceptibilities of 171 *A. fumigatus* isolates were examined, most of them were found to be susceptible to antifungal agents when they were not forming biofilms.

In contrast, biofilm-forming *A. fumigatus* showed resistance to most antifungal agents, except the combination of micafungin and amphotericin B, which suggested synergism.

My study showed that the serum have substantial effect on biofilm formation and the degree of ECM formation varies significantly depending on the *Aspergillus* species. Since this biofilm-forming capacity has been suspected of being an important tool for the persistence of infection, my finding is of particular interest from a clinical aspect. Although some other factors
such as gliotoxin production have been proposed as an important virulence factor, no single factor has been shown to play a key role in the pathogenesis. As shown in this research, the biofilm-forming capacity of *A. fumigatus* shown here could be another candidate for its virulence property, and an analysis of the production of thick ECM and its structure will shed light on the development of new therapeutic targets. From the therapeutic point of view, a combination of antifungal agents was suggested to carry a potential for the treatment of this intractable biofilm disease.