Original Article

Histopathological Examination of in vitro Bone Degeneration Caused by a Black Yeast, *Exophiala spinifera*

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Abstract

*Exophiala spinifera*, a black yeast, rarely causes systemic infection, and only a very few cases of its infection by the invasion of internal organs or bones have been reported. We examined the ability of *E. spinifera* to invade bone tissues in vitro. The fungus was inoculated on the surface of murine bones, and then these bones were incubated at 30°C for 2, 4, and 12 weeks on water agar plates and on brain heart infusion agar supplemented with 1% glucose (BHIA) plates. Histopathological examination demonstrated that the fungus was initially found in the non-calcified parts of the bone tissue, such as the growth plate and articular cartilage. Thereafter, the fungus invaded the calcified parts: cancellous and cortical bones. Our experiments showed that the capability of *E. spinifera* to invade bone tissue is higher than that of *Candida albicans* or other black fungi. *E. spinifera* grew in the mycelial form and *C. albicans* in the yeast form in these experiments. Our results suggest that *E. spinifera* may have a high potential to invade bone tissues, and that the mycelial form can invade bone more deeply than the yeast form. Therefore, bone degeneration should/must be carefully monitored in any systemic infection with *E. spinifera*.

Key words: *Exophiala spinifera*, bone, degeneration

Introduction

A lesion destroying the bone is observed in patients with tuberculosis caused by *Mycobacterium tuberculosis*¹, and with syphilis by *Treponema pallidum*². However, this bone destruction has been regarded as a secondary effect of inflammation by those bacteria. Actually, it is not common for some species of bacteria to be directly involved in the inflammation of osteolysis or bone destruction. *Streptococcus mitis* is one of the rare species responsible for bone destruction, and it produces a cytopathic toxin that induces morphological changes in osteoblastic cells³. Fungi that cause severe mycosis such as blastomycosis, chromomycosis, coccidioidomycosis, paracoccidioidomycosis, or sporotrichosis are also known to induce inflammation with bone destruction⁴. *Coccidioides immitis* is a representative sample, and it causes bone destruction in a patient who has an abscess around the spine⁵. This report suggests that some human pathogenic fungi, as well as *C. immitis*, may cause inflammation with bone destruction after fungal invasion. *Exophiala spinifera* was originally isolated from a solitary granulomatous mass of the right nasal septum in a patient under treatment for tuberculosis⁶. Furthermore, in two cases of *E. spinifera* disease reported by Campos-Takaki and Jardim⁷, and Li and Wang⁸, bone degeneration was found in patients showing disseminated infection. Histopathological examination of bone tissues was not well described in these reports however. To confirm bone destruction caused by *E. spinifera* histopathologically, in vitro experiments were designed by inoculating the fungus on the surface of a murine bone.

Materials and Methods

In the present study, we used 3 strains of *E. spinifera* (IFM 41505 and 47058 from patients, IFM 46500 from soil; Table 1). Strains of *Candida albicans* IFM 40009, *Exophiala dermatitidis* IFM 4827 and *Fonsecaea pedrosoi* IFM 47060 were also

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used as reference species (Table 1). All strains were maintained by the Research Center for Pathogenic Fungi and Microbial Toxicoses, Chiba University. The strains were cultured at 25°C for 4 days on slants of brain heart infusion agar supplemented with 1% glucose (BHIA), except *F. pedrosoi* which was cultured for 7 days due to its slow growth.

The periosteum of the bone from limbs of 5 to 7 week-old mice (strain, ddY; SLC Co., Shizuoka, Japan) was cut off under a sterile condition, and received 10^8 yeast cells/ml of *E. spinifera* and *C. albicans* on the surface of epiphysis and diaphysis of bone, individually. These samples were then cultured at 30°C for 2, 4, and 12 weeks on 1.5% water agar (WA) plates as poor nutrient medium, and on BHIA plates as nutrient rich medium. On the other hand, *F. pedrosoi* and *E. dermatitidis* were cultured at 30°C for 4 weeks on WA and BHIA plates.

Each of the bones cultured for a defined period was fixed in 10% formalin for 24 hrs, demineralized with Plank-Rychlo's mixture for 3 days, and then embedded in paraffin. Sections of the bone, 8 μm in thickness, were prepared by conventional procedure and stained with periodic acid-Schiff (PAS) reaction for microscopic observation.

### Results

Three strains of *E. spinifera* showed intraosseous invasion into murine bone, but no significant differences in invasion among the 3 strains were observed (Table 2). At week 2 after culture on WA plate, the fungus was found in the sites of the growth plates, articular cartilage, cancellous bone, cortical bone, and bone marrow (Fig. 1; Table 2), and at weeks 4 and 12 the fungus had extended to deeper sites of the articular cartilage (Fig. 2), cancellous bone, cortical bone (Figs. 3, 4) and bone marrow (data not shown). The fungal cells observed were the mycelial form on the

<table>
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<th>Strain No.</th>
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<th>2 weeks culture</th>
<th>4 weeks culture</th>
<th>12 weeks culture</th>
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+ + + : severe degree of fungal elements.
+- + : moderate degree of fungal elements.
+- : mild degree of fungal elements.
- : fungal elements were not observed.
ND: not done.
surface of bone and at intraosseous tissues. On BHIA plate, E. spinifera was not observed in the sites of cancellous or cortical bone after 4 weeks of culture, and thus the fungal invasion was limited compared with the culture on WA plate (Table 2). The fungal cells were the yeast form on the surface of bones, and both yeast and mycelial forms in intraosseous tissues.

In the case of C. albicans grown on WA plate, the fungus was found in the site of the growth plate at week 2, and developed to growth plates and cancellous bone at week 4, then at week 12 also it invaded the articular cartilage. On BHIA, C. albicans was not observed in intraosseous tissues at either 2 or 4 weeks (Fig. 5). At week 12, the fungus was found in the growth plate and articular cartilage as yeast cells, and occasionally as pseudomycelial cells.

F. pedrosoi and E. dermatitidis were noted in the sites of the growth plate, articular cartilage, and cancellous bone after culture for 4 weeks on WA plate. On BHIA, they were found only in the site of the growth plate. F. pedrosoi was characterized as mycelial cells on the surface of bone and in intraosseous tissues in cultured on both plates, but E. dermatitidis, was demonstrated yeast cells on the surface of bone on BHIA (Table 2).

Discussion

A pathogenic dematiaceous fungus, E. spinifera causes a severe invasive mycosis. In this disease, the fungus colonizes not only in a subcutaneous lesion but also bone tissue. In the present study, we examined whether E. spinifera seeded
on the surface of murine bone causes bone degeneration. As shown in Table 2, the fungus was distributed in the sites of growth plates, articular cartilage, cancellous bone, cortical bone, and bone marrow when cultured on WA plate, whereas on BHIA its presence was limited to the growth plates and articular cartilage during 12 weeks of culture. This may be due to the rich nutrient medium of BHIA. In addition, *E. spinifera* showed mycelial cells when cultured on WA plates, while both mycelial and yeast cells were observed in the specimen cultured on BHIA plates. Only mycelial cells caused deep-seated invasion of bone tissues. The results obtained here suggested that the mycelial form of *E. spinifera* most easily invaded intraosseous tissues. And a poor nutrient WA medium produced mycelial cells in *E. spinifera*. The rich culture of BHIA medium might induce the formation of yeast cells instead of mycelial cells.

*C. albicans*, one of the most important pathogenic fungi, is a resident on the microflora of skin, oral cavity, and intestine. It is mainly yeast cells when cultured on WA and BHIA medium. Table 2 showed that pseudomycelium and yeast cells of *C. albicans* were found at the sites of growth plates and articular cartilage at week 4, and then reached cancellous bone at week 12, while after 12 weeks of culture only yeast cells were found in these sites. This suggests that pseudomycelial cells of *C. albicans* present in bone tissue for a long period may be involved in degeneration of bone.

Four weeks-culture of *F. pedrosoi* and *E. dermatitidis* on WA resulted in the deep seated invasion of bone along with the formation of mycelial cells, but that on BHIA did not, even though mycelial cells were produced. Thus, in an enriched condition these strains may lose the ability to colonize in deep sites of bone tissue.

In conclusion, we demonstrated that *E. spinifera* has the ability to invade intraosseous tissues, and its formation of mycelial cells may colonize in deep sites such as cortical bone and bone marrow. Therefore, bone degeneration is a factor to be watched for in patients showing *E. spinifera* infection.

Acknowledgement

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References