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Original Article

Direct Invasion of Bones by Highly Pathogenic Fungi in an *in vitro* Model and Its Ecological Significance

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Abstract

Animal bones after being devitalized at death are strongly resistant to wear and tear and remain in the soil or environment much longer than other organic components from dead animals. Yet over the course of time they seem to disappear and thus our ecological surroundings are not cluttered with bone remnants. Mechanical factors creating compression or friction and chemical factors like pH of the soil and surroundings must together have provided concerted degrading effects. Microorganisms in the soil also help in this process by utilizing the organic components of devitalized bones. Certain highly pathogenic fungi that have been collected from soil from time to time and many other environmental fungi may take part in the degrading of the bone remnants. In this study, several strains from the highly pathogenic dimorphic fungi *Coccidioides immitis*, *Blastomyces dermatitidis*, *Histoplasma* spp., *Paracoccidioides brasiliensis* and also some strains of dematiaceous fungi (*Exophiala* spp. and *Foncecaea pedrosoi*) were inoculated to dissected and devitalized murine long bones that had been placed on solidified water agar plates to see if they would survive, grow and invade the bones. After being kept for 12 weeks at 25°C all the parts of the histological sections of these bones showed invasion by most of the strains used in this study, although the cortical component of the bony architecture seemed to be comparatively resistant to invasion. Their ability to grow and sporulate in the aforementioned nutrient-limiting condition hinted at a possible role of these fungi in the degradation of devitalized bones.

Key words: bones, invasion, environment, thermo-dependent dimorphic fungi, dematiaceous fungi.

Introduction

A clear understanding regarding the natural destruction and ecological transformation of bones after the death of animals is lacking. Bones have evolved as special supporting structures in vertebrate animals and are composed mainly of the inorganic mineral hydroxyapatite. Other minerals such as magnesium, sodium and potassium are also present in small quantities. The organic component of bones comprises a type I collagen and small amounts of proteoglycans and proteins. In life, the bone is a dynamic organ that perpetually undergoes the processes of remodeling and resorption executed by two groups of cells, the osteoblasts of mesenchymal origin and the osteoclasts of myeloid origin. Recently, it has been proposed by some researchers that in conditions of both health and disease bone remodeling and resorption take place in two steps: the first step is driven by the osteoclasts causing acid dissolution and destruction of hydroxyapatite and as the second step the tissue macrophages or giant cells provide an acidic collagenolytic protein that causes destruction of the type I collagen[1, 2]. Whereas in good health the balance between these two steps is optimum and finely tuned, it is uncontrolled in various pathological conditions of the bone.

In the environmental context, the bones of dead animals are destroyed by a different mechanism; here obviously, in the absence of macrophage-function, the organic component is utilized first by different environmental microorganisms

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Table 1. Fungal isolates used in this study

Species	Strain no.	Remarks
Coccidioides immitis	IFM 4935	Patient, Nagoya Univ. Japan
	IFM 4945	Patient, Toho Univ. Japan
	IFM 45809	Patient, San Jose, USA
	IFM 45810	Patient, San Jose, USA
	IFM 45811	Patient, San Jose, USA
	IFM 45812	Patient, San Jose, USA
	IFM 45813	Patient, San Jose, USA
	IFM 45815	Patient, San Jose, USA
	IFM 45816	Patient, San Jose, USA
	IFM 45817	Patient, San Jose, USA
	IFM 45868	Patient, Yokohama, Japan
	IFM 50992	Patient, USA
	IFM 50993	Japanese Patient who lived in Arizona for 2 years
	IFM 50994	Patient, Japan
	IFM 50995	Patient, Japan
Blastomyces dermatitidis	IFM 41314	ATCC 26197
	IFM 41315	ATCC 26198
	IFM 41316	ATCC 26199
	IFM 41317	Patient, CDC, A-295
	IFM 41318	Patient, Emory Univ. USA
H. cap. var. *capsulatum*	IFM 5396	Hi-1, MTU 16001
	IFM 41329	Patient, CDC 105
	IFM 47750	Vietnamese patient, Japan
	IFM 49109	Patient, Japan
	IFM 49721	Patient, Brazil
H. cap. var. *duboisii*	IFM 5416	Hi-d-2
	IFM 5417	Hi-d-3
	IFM 41332	CDC B-650
	IFM 50954	An Ugandan patient
H. cap. var. *farciminosum*	IFM 5418	Hi-f-1
	IFM 41333	SM 1024
	IFM 41334	CDC B-2218
	IFM 41335	CDC B-2218
	IFM 41612	Patient, Japan
P. brasiliensis	IFM 41620 (Pb-9)	Patient, Sao Paulo Univ. Brazil
	IFM 41621 (Pb-18)	Patient, Sao Paulo Univ. Brazil
	IFM 41624 (Bt-4)	Patient, Botucatu Univ. Brazil
	IFM 41625 (Bt-7)	Patient, Botucatu Univ. Brazil
	IFM 41626 (Bt-9)	Patient, Botucatu Univ. Brazil
	IFM 41628 (B-1183)	Patient, CDC
	IFM 41630 (Pb-339)	Patient, Campinas Univ. Brazil
	IFM 41632 (Pb-HM-AOKI)	Patient, Japan
	IFM 41633 (Hachisuga)	Patient, Kurume Univ. Japan
	IFM 46215 (Wagura)	Patient, Japan
	IFM 46463 (Tatu)	Armadillo, Brazil
	IFM 47183 (Tatu-1)	Armadillo spleen, Brazil
	IFM 47195 (D3LY1)	Armadillo lymph node, Brazil
	IFM 47207 (D3S1)	Armadillo spleen, Brazil
	IFM 47217 (D4S1)	Armadillo spleen, Brazil
	IFM 49627 (Pb Maciel Ortiz)	Patient, Argentina
Sporothrix schenckii	IFM 41598	Patient, pulmonary sporotrichosis, CUCM
	IFM 46927	Patient, Japan
	IFM 47068	Patient, skin, Japan
	IFM 47069	Patient, Japan
	IFM 50225	Patient, skin, Japan
	IFM 50545	Patient, skin, Japan
Exophiala dermatitidis	IFM 4827	CBS 207.35
Fonsecaea pedrosoi	IFM 47060	Patient, skin, Japan
Exophiala spinifera	IFM 41505	Patient, USA

and various other factors of the food chain. The remaining inorganic component of bones is quite resistant to wear and tear by natural processes, but that also weakens over time so our environment is not cluttered with bones of the dead. The agents or factors responsible for this degradation process are still unclear. Chemical, mechanical and physical factors in nature seem to be strongly implicated although evidence of any biological agent coming directly into play is still

Table 2. Invasion status of bone by fungi on water agar medium at 12 weeks

Fungal species	Growth plate	Cancellous bone		Articular cartilage		Cortical bone		
		[a]SA	[b]SGP	[c]R	[b]S	[c]SE	[f]VO	[g]LS
Coccidioides immitis	+	+	+	+	−	−	+	−
Blastomyces dermatitidis	+	+	−	+	+	+	+	+
Histoplasma capsulatum var. *capsulatum*	+	+	−	+	−	−	+	−
H. capsulatum var. *duboisii*	+	+	−	+	+	−	+	−
H. capsulatum var. *farciminosum*	+	+	−	+	+	+	+	−
Paracoccidioides brasiliensis	+	+	−	+	−	−	+	−
Sporothrix schenckii	+	+	+	+	+	−	+	−
Exophiala dermatitidis	+	+	+	+	+	+	+	+
Fonsecaea pedrosoi	+	+	+	+	+	+	+	+
Exophiala spinifera	+	+	+	+	+	+	+	+

[a]SA: sub-articular [b]SGP: sub-growth plate
[c]R: rough [d]S: smooth
[e]SE: surface erosion [f]VO: vascular opening
[g]LS: lamellar separation

lacking; in nature the effects of fungi on scattered dead bones is still unclear. Despite the shorter incubation period of our *in vitro* experiment compared to the much longer time-expanse of the natural degradation process which occurs in nature, most strains showed promising degrees of invasiveness to all parts of the bone by the abundant presence of hyphae and conidia. The fungal isolates exhausted the architecture of compact cortical bones by creating irregular cratered surfaces and causing impingement. The sections showed extensive invasion in all parts of bones by both the dematiaceous and the thermo-dependent dimorphic fungi. Moreover, recent observations from this group strongly implicated at a role for certain black fungi in the ecological breakdown of devitalized bone substrates[3].

This study is aimed at identifying the possible role of several pathogenic thermo-dependent dimorphic fungi and black yeast[4, 5] in the ecological destruction of devitalized bones.

Materials and Methods

Isolates

Fifteen isolates of *Coccidioides immitis*, 5 of *Blastomyces dermatitidis*, 5 of *Histoplasma capsulatum* var. *capsulatum*, 4 of *H. capsulatum* var. *duboisii*, 5 of *H. capsulatum* var. *farciminosum*, 16 of *Paracoccidioides brasiliensis* and 6 of *Sporothrix schenckii* were used in this study to observe their potential for destroying devitalized bones. An isolate from each of the black fungi, *Exophiala dermatitidis*, *Fonsecaea pedrosoi* and *Exophiala spinifera* was also included in this study. All the above strains are stored and maintained in the culture collection of this laboratory (Table 1).

Culture

All isolates of thermo-dependent dimorphic fungi were grown on slants of brain heart infusion (BHI, Becton Dickinson Microbiology Systems, Sparks, MD, USA) in 1.5% agar supplemented with 1% dextrose at 35℃ for 1 week. The isolates of *S. schenckii*, *E. dermatitidis*, *F. pedrosoi* and *E. spinifera* were grown on potato dextrose agar (PDA, Difco, Detroit, USA) slants at 25℃ for 1 week. The fungal isolates were implanted on surfaces of aseptically excised bones laid on plastic petri dishes (90 × 20 mm in diameter) containing approximately 30 m*l* of solidified water agar (1.5%) incorporated with 0.01% chloramphenicol (Wako Pure Chemicals, Osaka, Japan) to prevent the growth of unwanted spurious microorganisms. The bone periosteum was not removed. The petri dish cultures were maintained at 25℃ for 12 weeks. The limb bones were obtained from fifty-one male mice approximately 6-8 weeks old, (strain ddY, SLC Co., Shizuoka, Japan). The mice were fed a normal laboratory diet, kept at 25±1℃ temperature with 55±5% humidity and were provided with clean drinking water *ad libitum*. They were sacrificed by cervical dislocation under ether anesthesia for this experiment. This work complied with all relevant guidelines and policies of the Animal Welfare Committee of the Faculty of Medicine of Chiba University, Japan.

Histological processing

After allowing the fungal inoculates to grow for 12 weeks on excised bones, the fungal cells were killed and fixed with 4% formaldehyde treatment for 24 hours. The bones were then taken from the petri dishes, fixed again in 4% formaldehyde and decalcified in Plank-Rychlo solution[6]. Automated tissue processing was carried out by routine procedure on a rotary machine containing gradations of alcohol, xylene and paraffin before the bones were finally embedded in paraffin, then cut into 4-6 μm thick sections. The histological

sections were stained by hematoxylin-eosin (H/E) and the periodic acid-Schiff (PAS) method with hematoxylin counterstaining. They were microscopically examined under a light microscope to determine the extent of fungal invasion to the bone structures.

Results

All five histological landmarks of a long bone: the articular cartilage, cancellous bone, growth plate, bone marrow and the cortical bone were minutely observed under the microscope for any fungal presence. The fungal isolates tested in the present study demonstrated filamentous forms and sporulation in and around the bone structures and all stained a bright magenta color due to the periodic acid-Schiff reaction of fungal cell wall components. Irrespective of the fungal species or the isolates that were laid on the bones, maximum fungal presence was seen in the bone marrow compartments. Fungal entrance was facilitated by the openings of vascular pores. In the case of growth plate invasion, extension was seen to be progress from the sides, possibly by utilizing decomposing organic substances present along reminiscent vascular structures and then permeating inwards from the sides. Cancellous bones on both sides of the growth plate were invaded by fungal elements; articular cartilages were invaded to varying degrees by the different strains used in this study. In cancellous bones, fungal invasion was more severe under the articular cartilages than under the growth plates. In the articular cartilages, fungal access was more prominent at the roughened borders for ligamentous and tendinous insertions than at the smooth and central cartilaginous part. The least presence of fungal cells was seen in cortical bones, which were only sparsely invaded compared to other parts of the bone. The respective invasiveness of each fungal species on different parts of bone is shown in Table 2.

The strains of *C. immitis* invaded all bone parts and in Fig. 1a. the sub-growth plate cancellous bone section is shown with abundant filamentous forms of the fungi. The roughened borders of the articular cartilages and the vascular openings at the cortical parts showed fungal invasion. Arthroconidia were observed outside the bones. The strains of *B. dermatitidis* (Fig. 1b.) used invaded the growth plates, the sub-articular part of cancellous bones and both the rough and smooth parts of articular cartilages; they also grossly invaded the cortical bone causing lamellar separation along the long axis. There was evidence of surface erosion along with fungal aggregations at vascular openings of cortical parts, and chlamydospore formation was observed outside the bones. Some fungal masses composed of intermediate forms that were between yeast cells and chlamydospores were attached to the articular

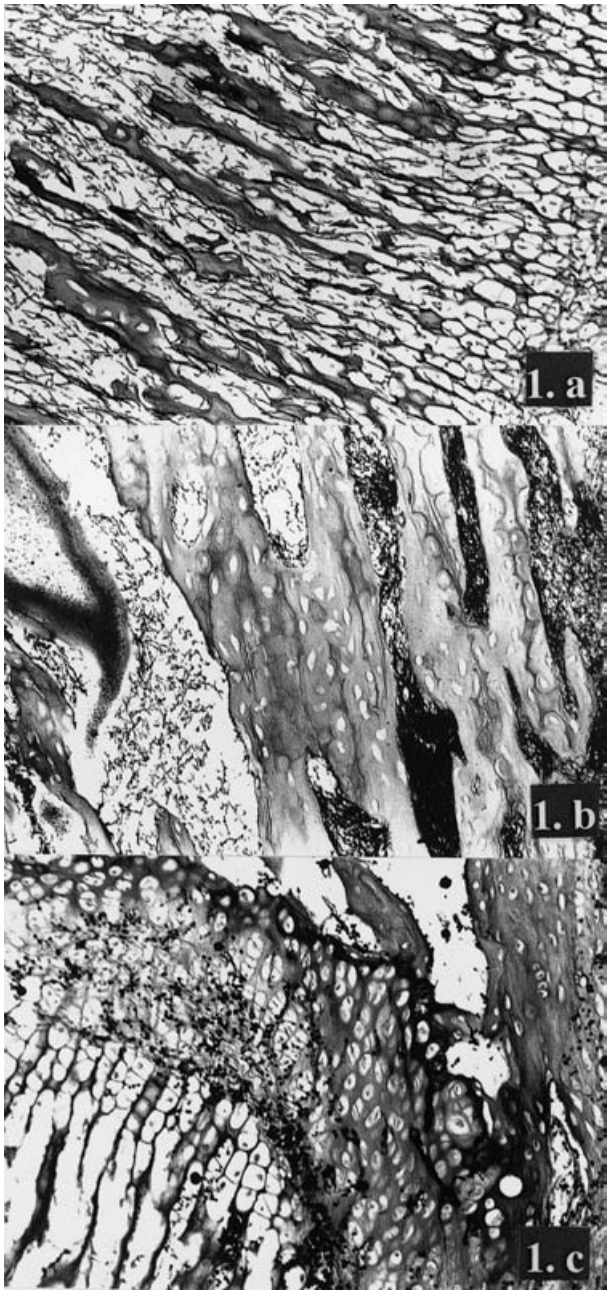

Fig 1. Parts of bone invaded by *C. immitis*, *B. dermatitidis* and *H. capsulatum* var. *duboisii* are shown. The bone specimens were stained by the PAS reaction and counterstained with hematoxylin. Observed by a light microscope at 150 times magnification. a) *C. immitis* (IFM 50994 strain) has invaded the sub-growth plate cancellous part; a network of mycelial growth-expansions appear to narrow down these cancellous parts. b) *B. dermatitidis* (IFM 41316 strain) has extensively invaded the cortical part; lamellar separation along the long axis of the bone are visible. c) *H. capsulatum* var. *duboisii* (IFM 50954 strain) has invaded the growth plate region; micro- and macroconidia can be seen.

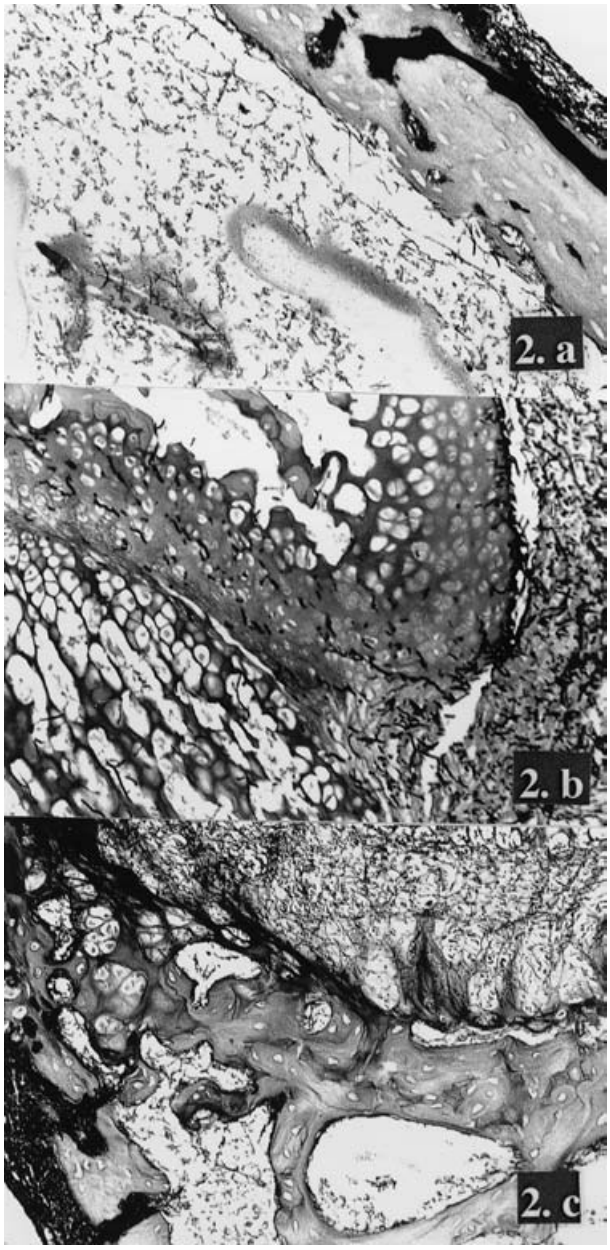

Fig 2. Parts of bone invaded by *H. cap.* var. *farciminosum*, *F. pedrosoi* and *E. spinifera*. The bone specimens were stained by the PAS reaction and counterstained with hematoxylin. Observed by a light microscope at 150 times magnification. a) *H. cap.* var. *farciminosum* (IFM 41335 strain) has invaded the cortical part of long bones and surface erosions are also seen. The vascular pores are filled with fungal growth. b) *F. pedrosoi* (IFM 47060 strain) invading the articular cartilage. The dark elongated structures seen penetrating the cartilage borders are fungal elements. c) *E. spinifera* (IFM 41505 strain) invading the cancellous part of long bones. In the vacuolar spaces of cancellous bones fungal growth can be observed.

cartilage surfaces. In the case of *H. capsulatum* var. *capsulatum* only the sub-articular cancellous, the rough articular and the vascular channel pores along with the growth plate and bone marrow were invaded. *H. capsulatum* var. *duboisii* strains invaded to a similar degree and were also observed in the smooth part of the articular cartilage but no surface erosions of cortical bone were observed. In Fig. 1c. the growth plate is shown to be invaded by macro- and microconidia. The smooth surface of the articular cartilage was also invaded and surface erosions at the cortical bones were seen in cases of *H. capsulatum* var. *farciminosum* strains as shown in Fig. 2a. The strains of *P. brasiliensis* showed similar levels of invasiveness as *H. capsulatum* var. *capsulatum*; aleurioconidia or chlamydospores were observed in the cancellous bone between the articular cartilage and the growth plate. The last dimorphic fungus in this study, which is also a black yeast, *S. schenckii*, was found to invade all bony parts except the cortical bones where only the vascular pores were invaded.

All the other three dematiaceous fungal (*E. dermatitidis*, *F. pedrosoi* and *E. spinifera*) strains displayed extensive invasiveness in all parts of the bone; conidia formation was observed everywhere, mainly in the bone marrow compartment. In Fig. 2. b., invasion of the articular cartilage by *F. pedrosoi* is shown; both the smooth cartilaginous and the rough corners were invaded by the fungi. In Fig. 2. c. the sub-growth plate cancellous bone is shown being invaded by *E. spinifera* and conidia formation is observed in the bone marrow compartment and also outside the bones. The cortical bones exhibited erosions caused by *H. capsulatum* var. *farciminosum* on both the outer and inner surfaces. In addition to fungal abundance on external sides of bones and in the bone marrow compartment, the vascular channel pores were filled with aggregates of fungal growth.

Discussion

Bone pathology caused directly and indirectly[7,8] by various bacteria or fungi[9] is known. The living bone is a dynamic organ that undergoes perpetual resorption and remodeling by bone cells: the osteoblasts, the osteoclasts and the monocyte-macrophage system. But in dead or devitalized bones, most of these components no longer exist or may only exist initially, as decomposing organic materials that are soon to be utilized by various soil and environmental microorganisms. The remaining bone structure is mainly a framework of inorganic components, and although it takes much longer, it too is finally degraded; thus over the past millions of years since the evolution of vertebrate animals, our ecological surroundings have not become cluttered with incompletely degraded bone remnants. Over the course of time these remnants have degraded and possibly been

recycled in the food chain by numerous agents. Various human pathogenic fungi are known to have environmental niches in their life cycles and it has also been observed that instead of direct human-to-human transmission, a passage through soil or the environment occurs before fungi regain the virulence factors necessary for their pathogenic potential to animals. It has been postulated by us that devitalized bones scattered in the soil might serve as prospective environmental niches for these pathogenic fungi. The present study clearly demonstrated that most of these pathogenic fungi (both the thermo-dependent dimorphic and the dematiaceous) might very well grow and produce inside devitalized bones. In the process, resorption and loss of bones were evidenced. Aggregates of fungal growth were also seen creating shearing pressure within hard cortical bones and thus opening up their way through it.

The cortical parts of bones seemed most resistant to fungal invasion by all strains used in this study. Primarily, three types of cortical bone damage were observed here: 1) surface erosions on one or both sides of these bones and fungal presence at and near erosion sites; 2) common to all strains was the presence of fungal aggregates inside the vascular openings; and, 3) although rare and seen in only a few strains, the separation of the cortical bone along its long axis into lamellar, pitted structures. In all cases the maximum fungal presence was seen in the bone marrow where entry may have been easily accomplished through the vascular pores and fungal growth and proliferation facilitated by the abundance of decomposing organic nutrients occurred there. Interestingly, all fungi tested produced some form of conidia or spores inside or outside the bones; this phenomenon indicated the possible natures of infectious propagules of these pathogenic fungi. As shown under laboratory culture conditions, *H. capsulatum* species produced macro- and microconidia, the dematiaceous fungi produced pigmented conidia and *C. immitis* produced arthroconidia. *B. dermatitidis*, on the other hand, produced chlamydospores or yeast-like structures, and this group caused marked invasion of cortical bones causing lamellar separation along the long axis. *B. dermatitidis* forms blastospores at 37℃ on appropriate medium and these are presumed to be the infective element forms[10], although findings from this study indicate that the natural infectious propagule of *B. dermatitidis* may present as chlamydospores or yeast-like cells. *P. brasiliensis* formed chlamydospores and aleurioconidia in the bone marrow which may be potential forms of infectious propagules, although Brummer *et al.* have reported that the arthroconidia forms were infectious to animals[11]. Thus this study can be used as an experimental model offering insights into the nature of the infectious propagules of various thermo-dependent dimorphic fungi.

In conclusion, the findings of this experiment lend support to the hypothesis that fungi can survive and thrive inside devitalized bones in the environment and thereby take part in the gradual clearance of bone residue from soil. Further investigations in environmental models on how fungi degrade bones, the enzymes involved and the end products that result from this degradation will clarify this role.

Acknowledgments

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