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HYPOXIA SENSING IN *CRYPTOCOCCUS NEOFORMANS*: BIOFILM-LIKE ADAPTATION FOR DORMANCY?

Zuzana Moranova[a, b], Susumu Kawamoto[b], Vladislav Raclavsky[a]*

[a] *Department of Microbiology, Faculty of Medicine and Dentistry, Palacky University, Hnevotinska 3, 775 15 Olomouc, Czech Republic*
[b] *Medical Mycology Research Center, Chiba University, 1-8-1 Inohana, Chuo-ku, Chiba 260-8673, Japan*
e-mail: vladislav.raclavsky@upol.cz

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Background: *Cryptococcus neoformans* is an obligate aerobic pathogenic yeast causing lung infection typically followed by spread to the central nervous system. During pathogenesis, it relies on well-established virulence factors. This review focuses on the emerging role of cryptococcal adaptation to hypoxia in pathogenesis.

Methods and Results: We examined the MedLine database for information on the cryptococcal hypoxia response. While several recent papers describe components of two presumable hypoxia-sensing pathways including description of their target genes, a link of this system to the hypoxic tuning of proliferation is still missing. In addition, an interpretation of this knowledge in respect to the general picture of microbial pathogenesis is lacking.

Conclusions: There seems to be a striking parallel between biofilm formation in bacteria, which results in chronic dormant infection with the potential for acute outbreaks, and the dormant state of primary infection followed by secondary outbreaks in *C. neoformans*. We propose a hypothesis that cryptococcal response to hypoxia might be the driving force for developing a state of dormant infection which is characterized by slowed proliferation and extensive changes in transcriptome and phenotype. This state enables *C. neoformans* to survive in host and possibly develop life-threatening acute outbreaks later. Hence, conventional well-aerated planktonic culture is not a good *in vitro* model for studying the pathogenesis of infection and we advocate the development of a more adequate model. Our further conclusion is that the ability of the immune system and antifungal agents to cope with hypoxia-adapted cells is crucial for the successful eradication of cryptococcal infection.

INTRODUCTION

Cryptococcus neoformans is a heterothallic, basidiomycetous, pathogenic fungus that predominantly infects immunocompromised patients. The prevalence of disease caused by this organism has increased dramatically as a result of human immunodeficiency virus (HIV) infection, organ transplantation, cytotoxic chemotherapy and corticosteroid use.

There are three varieties of the fungus divided into four serotypes, which differ in ecological, morphological-physiological and molecular features, geographical distribution, epidemiology and virulence properties. Recently, some authors have suggested establishing *C. neoformans* var. *gattii* as a new species *C. gattii*, whereas *C. neoformans* var. *neoformans* and *C. neoformans* var. *grubii* should form the species *C. neoformans* sensu stricto[1]. *Cryptococcus neoformans* var. *grubii* (serotype A) and *C. neoformans* var. *neoformans* (serotype D) cause disease mainly in immunocompromised patients, whereas *C. neoformans* var. *gattii* (serotypes B, C) can also cause disease in immunocompetent patients. Most clinical and environmental isolates belong to serotype A.

Soil contaminated with bird (mainly pigeon) excreta was traditionally known as the natural habitat of *Cryptococcus neoformans* var. *grubii* and var. *neoformans*[2]. Later, high concentrations of the organism were also found in woody debris in the hollows of aged trees[3-5]. *C. neoformans* can utilize this woody material which is rich in lignin, polyphenol compounds and cellulose as a substrate for growth due to its laccase, phenol oxidase and cellulase activities[6]. Whereas *Cryptococcus neoformans* var. *grubii* and var. *neoformans* are ubiquitous, *Cryptococcus neoformans* var. *gattii* can be primarily found in tropical and subtropical regions where it is associated mainly with eucalyptus trees[7]. As nothing indicates that there are significant differences in oxygen signaling between *C. neoformans* and *C. gattii*, we will further simply refer to *C. neoformans* sensu lato, regardless whether it is a species complex or a complex species.

To cause disease, *C. neoformans* must adapt to the host environment which is very different to its natural habitat. *C. neoformans* has three well established virulence factors: a capsule, melanin and the ability to grow at human body temperature[8]. Capsule synthesis is induced by serum, iron deprivation and physiological carbon dioxide levels; it interferes with phagocytosis by macrophages and depletes complement components, thereby interfering with the host immune response. Acapsular mutants are attenuated in virulence. Melanin serves as an ultraviolet radiation protecting agent. It is formed by the oxidation of host catecholamines and protects the fungus from toxic free

radicals used by immune cells for killing infecting organisms. The ability to grow at human body temperature is a general condition for all human pathogens. In this review, we focus on the emerging role of cryptococcal ability to adapt to decreased oxygen levels during pathogenesis.

HYPOXIA SENSING

The ability to maintain dioxygen homeostasis is essential for survival of aerobic organisms. Oxygen is necessary for energy generation and also for some biosynthetic pathways (ascorbic acid, sterols, unsaturated fatty acids and heme). All aerobic species thus had to develop a strategy to cope with fluctuations in oxygen availability and to survive oxygen limitation. Therefore, they sense oxygen concentrations and respond to its different levels with adaptive changes in their phenotypes. Sensitivity to low oxygen levels varies not only in different organisms but also in different cell types. It is well known that human fungal pathogens require molecular oxygen for several essential biochemical reactions but there is a lack of information on the effect of hypoxia on infectivity and virulence of microbial pathogens and little is known how pathogens respond to hypoxia in the host environment[9].

Cryptococcus neoformans cell cycle progression is sensitive to different aeration

C. neoformans is an obligate aerobic pathogenic yeast for which oxygen is a growth-limiting nutrient as shown by Odds *et al.*[10]. These authors demonstrated that the growth yield of *C. neoformans* in microbroth cultures with RPMI 1640 medium was unaffected by glucose concentration and other potential carbon, nitrogen, and vitamin sources and that oxygen was the limiting nutrient for growth in microtiter plates. Agitation and 100% oxygen environment resulted in the highest OD readings. Rodriuez-Tudela *et al.* also noted that addition of glucose to RPMI medium to 2 % did not significantly improve *cryptococcal* growth[11]. Also Roberts *et al.* have shown that higher glucose concentration has little influence on *cryptococcal* yields compared to oxygen levels[12]. They also demonstrated that the isolation rate of *C. neoformans* from blood cultures was significantly enhanced by venting vacuum blood culture bottles in studies of both simulated and patients' blood cultures compared to other yeast species[12]. Later, Huahua *et al.* demonstrated that addition of hydrogen peroxide to blood culture bottles increased pO_2 levels which resulted in increased CFU/ml yields of *C. neoformans* compared to unvented and vented bottles[13]. Clinical isolates of *C. albicans* and *C. neoformans* which have been passaged several times in the laboratory grew significantly better in the bottles to which hydrogen peroxide had been added[13]. Taken together, such sensitivity to oxygen levels must rely on a delicate oxygen sensing system which controls cell proliferation.

Key findings pointing towards a better understanding of adaptation to hypoxia in proliferating cryptococcal cells came from cell cycle studies. Takeo *et al.*[14] first observed, that G1 as well as unbudded G2 cells are present in its stationary phase. Thus, *C. neoformans* is able to undergo DNA-replication without starting to bud, a feature making it quite different from the model yeast *S. cerevisiae*. The population of unbudded G2 cells was shown to result from gradually delayed onset of budding from S to G2, resulting in unbudded G2-arrest in some strains[15]. Finally, Ohkusu *et al.* identified deficit in oxygen under limited aeration as the cause for delayed budding resulting eventually in the unbudded G2-arrest[16]. Our observations indicate that oxygen can also become a limiting factor in a well-aerated submerged culture temporarily, i.e. during the late log phase of growth, when the number of cells increases rapidly and their oxygen consumption is very high (unpublished data). Sensing such limitation can enable the yeast to slow down proliferation temporarily to restore equilibrium in oxygen supply and consumption, exploit all the nutrients available and, eventually, arrest in response to nutrient limitation in G1. Strikingly different growth patterns were observed under different amount and intensity of aeration brought about by different volumes of submerged culture, agitation speed and types of sealing[17]. These data, together with low survival rate under long-term oxygen limitation[17, 18], indicate that the central strategy of *C. neoformans* lies in adjustment of its proliferation rate to the available oxygen levels and not in striving for survival under anoxia. In an attempt to exploit the differences in cell cycle regulation mechanisms of *C. neoformans* versus *Saccharomyces cerevisiae*, the cryptococcal CDC28 homologue CnCdk1 has been identified[19] and further studies are underway.

Adaptation to different oxygen levels appears to be important in pathogenesis of cryptococcosis

The importance of cryptococcal adaptation to low oxygen levels can be underlined by the fact that well ventilated lungs are the primary site of infection in this yeast species, typically followed by spread to the central nervous system (CNS), which represents a tissue with considerably lower albeit stable dissolved oxygen level[20]. Other organs are affected by cryptococcal infection rather rarely. It has been further demonstrated that during spread to CNS, cryptococcal cells stay in very close proximity to blood vessels when crossing the blood-brain barrier 3 hours after intravenous injection in a murine model of cryptococcosis[21]. Later they are able to invade brain parenchyma, however, still remain mainly close to capillaries. Large cystic lesions are formed only 10 days later; most of them again develop in close proximity to blood vessels[21]. Moreover, deletion of genes identified to be important for hypoxia signalling was shown to result in decreased virulence in a murine model of cryptococcosis[22, 23].

SREBP pathway seems to be at the center of hypoxia sensing in C. neoformans

Until recently, nothing was known about the molecular mechanisms underlying hypoxia response in *C. neoformans*. Most of our knowledge on oxygen sensing in yeasts refers to the model eukaryote *S. cerevisiae*. In this species,

oxygen sensing is mediated by heme. Heme biosynthesis is oxygen dependent and heme mediates the effect of oxygen on the expression of many genes involved in utilizing or detoxifying oxygen. Findings on the oxygen sensing system in *S. cerevisiae* can hardly be simply extrapolated to *C. neoformans*, since *S. cerevisiae* switches to a completely different metabolic behaviour in response to oxygen limitation and this enables it to continue its cell cycle progression.

New hints came surprisingly from studies on feedback regulation of cholesterol synthesis in animals. When defining the mechanisms that cells use to monitor changes in cholesterol levels and regulate lipid metabolism, the sterol regulatory element binding protein (SREBP) family of transcription factors was found to be at the centre of this feedback[24]. SREBP proteins are initially bound to the rough endoplasmic reticulum (ER) membrane and are regulated by sterols and SCAP (SREBP-cleavage activating protein). In the presence of cholesterol, SREBPs are sequestered in the ER. In the absence of signal, SREBPs undergo proteolytic events that lead to activation of genes that control lipid metabolism (for review see[25]). The SREBP pathway has been found to regulate a diverse set of cellular processes, including phagocytosis, cell cycle progression and survival in response to bacterial infection[26]. In mice, SREBPs are known to enhance directly the transcription of more than 30 genes needed for uptake and synthesis of cholesterol, fatty acids, triglycerides and phospholipids[27]. Following studies in animals, SREBP and SCAP homologues were also identified in the fission yeast *Schizosaccharomyces pombe* and demonstrated to play role in adaptation to hypoxia[28, 29]. Sre1 is cleaved and activated in response to sterol depletion in a Scp1-dependent manner and stimulates transcription of genes required for adaptation to hypoxia.

Later, Chun *et al.*[22] isolated hypoxia sensitive mutants harboring mutations in cryptococcal SREBP homologue Sre1, Site-2-protease homologue Stp1 and SCAP homologue Scp1. These mutants exhibit defects in growth under hypoxic conditions but not under normoxic conditions. The same group further identified a hypoxia-sensitive mutant in Tco1, a member of a highly conserved family of fungal-specific hybrid histidine kinases. Tco1 is required for virulence; it negatively regulates melanin formation and positively regulates the Hog1 MAPK pathway, which is dispensable for virulence[30]. In parallel with the work of Chun *et al.*[22], Chang *et al.*[23] isolated hypoxia sensitive mutants *sre1* and *scp1* and confirmed these observations. Tco1 and SREBPs pathways function in parallel, since the *tco1sre1* double mutant is more sensitive to hypoxia. Mutants in either the SREBP pathway or the Tco1 pathway display defects in their ability to proliferate in host tissues and to cause disease in infected mice, linking hypoxic adaptation and pathogenesis. Since Sre1 as well as Tco1 mutants grow normally in YPD as well in YNB medium under normoxic conditions, the defect in virulence is most probably not due to a general growth defect. Cryptococcal SRE1 was shown to be required for regulation of ergosterol synthesis through transcriptional control of oxygen dependent enzymes in the ergosterol biosynthetic pathway in response to low oxygen (all ref.[22]). In addition, cobalt chloride, a hypoxia-mimicking agent, was demonstrated to cause defects in several enzymatic steps in ergosterol biosynthesis which were amplified in cells lacking components of the Sre1p-mediated oxygen sensing pathway[31]. Significant changes in expression of genes involved in respiration and iron and sterol homeostasis, as well as ubiquitination were later observed in wild-type cells exposed to $CoCl_2$ and several mitochondrial functions were identified as critical for cryptococcal survival under low-oxygen conditions and $CoCl_2$ stress[32].

Future research prospects

In the near future, it should be clarified, whether the SREBP and Tco1 pathways are the only two sensing systems involved in hypoxia adaptation in *C. neoformans*. It also remains to be elucidated whether any of these pathways are also responsible for the hypoxia-induced unbudded G2-arrest or whether there are alternative systems controlling cell cycle machinery under hypoxic conditions. In mice, SREBP-1a protein was demonstrated to inhibit cell growth completely through accumulation of CDK inhibitors p21, p27 and p16, resulting in G1 cell cycle arrest[33, 34]. Although a G1-arrest is not consistent with the observation made in cryptococcal cells suffering hypoxia, partial conservation of signalling systems cannot be excluded. Because of the circumstantial evidence that the ability to adapt to low oxygen levels may play a key role in the ability of *C. neoformans* to spread from lungs to CNS, detailed knowledge of this molecular machinery can offer attractive targets for future anticryptococcal agents. Notably, SREBP pathway mutants were found to be a hundred times more sensitive than wild-type to fluconazole, an antifungal agent that inhibits ergosterol synthesis. This is well in accordance with the mechanism of action of fluconazole, which inhibits cytochrome P450 lanosterol 14-α demethylase, a key enzyme in ergosterol biosynthesis. Thus, malfunction of sterol synthesis in SREBP mutants obviously makes them hypersensitive to further insults to proper membrane synthesis by fluconazole. Because fluconazole and amphotericin B are the most widely used agents in the treatment of cryptococcosis, these observations suggest that inhibitors of SREBP processing could substantially enhance the potency of current therapies[22]. In addition, a plant-derived alkaloid sampangine was demonstrated to show antifungal activity towards *C. neoformans*, *C. albicans* and *Aspergillus fumigatus* that is most probably mediated through perturbations in the biosynthesis or metabolism of heme[35, 36]. This suggests an attractive possibility to target more than one vital oxygen-dependent process in fungal pathogens by combination of drugs for synergic action.

BEHIND THE CURTAIN OF HYPOXIA SENSING

Remarkably, changes in the levels of 347 transcripts were observed in *C. neoformans* in response to hypoxia[22].

This, together with the aeration-dependent fine tuning of cell cycle progression, can be highly relevant with respect to mechanisms of pathogenesis and practicable treatment strategies. There is a striking parallel between profound changes in both cryptococcal transcriptome and proliferation during adaptation to hypoxia, and the deep change in transcriptome and proliferation during bacterial biofilm formation. In bacteria, these adaptive changes result in biofilm cells which are clearly phenotypically distinct from their planktonic counterparts, resulting in an extraordinary increase in resistance to the action of both immune system and antibacterial agents (for review see[37, 38]). Typically, bacterial biofilm infections are chronic diseases threatening particularly immunocompromised patients by outbreaks of acute infection following detachment of bacteria from the primary site of biofilm formation.

In many cases of cryptococcosis, development of primary pulmonary lymph node complex is most probably followed by an asymptomatic dormant yeast infection which can be present for many years[39] and may be a source of secondary acute infection with a later immunosuppresive event[40] or possibly with phenotypic switching that increases the virulence of cryptococcal cells[41, 42]. When residing in such dormant state in tissue, the phenotype of cryptococcal cells should presumably be quite different from cells proliferating in well aerated and nutrient-rich laboratory cultures in many aspects relevant for treatment of infection, e.g. cell wall structure, plasma membrane composition, several aspects of metabolism etc. As already reviewed above, proliferation rate and transcriptome are subject to well documented profound changes under hypoxic conditions in *C. neoformans*. Furthermore, substantial overlap and interplay in gene regulation during hypoxic response and biofilm formation has been found in the yeast pathogens *C. albicans*[43, 44] and *C. parapsilosis*[45] recently, indicating that the foundations of these two adaptive strategies overlap and are conserved in different yeast species. Although *C. neoformans* is known to develop biofilm on artificial surfaces *in vitro*[46], there is no evidence for cryptococcal biofilm formation during infection of lungs or CNS, except for a case of shunt infection[47]. We therefore propose a hypothesis that adaptation to hypoxia may be the key driving force of the cryptococcal ability to enter a state of dormancy favourable for survival in host tissue. Very slow proliferation and a specific transcriptome should be the two main cornerstones of this strategy closely related to the biofilm strategy. Then, the ability of immune system and/or antifungal agents to cope with such dormant state cells should be crucial for the successful eradication of initial cryptococcal infection. When this eradication is not accomplished, immunocompromising insults or phenotypic switching present a long-term threat to the host, possibly resulting in a life threatening acute outbreak. Future diagnostic and treatment strategies and searches for new antifungal agents should bear this view in mind. In addition, establishment of an appropriate culture system to grow hypoxia-adapted "dormant" cryptococcal cells *in vitro* also remains an important challenge for future studies in this field because conventional well-aerated planktonic culture can hardly provide adequate data on cryptococcal physiology and antifungal susceptibility relevant in human infection[17].

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