Published in final edited form as:

Can J Microbiol. 2014 January ; 60(1): 1–4. doi:10.1139/cjm-2013-0708.

The Effects of Fungal Volatile Organic Compounds on Bone Marrow Stromal Cells

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Abstract

Evidence has shown that individuals exposed to indoor toxic molds for extended periods of time have elevated risk of developing numerous respiratory illnesses and certain types of cancer. It is not clear at the cellular level, what impact mold exposure has on the immune system. Herein we show that two fungal volatiles (*E*)-2-octenal and oct-1-en-3-ol have cytotoxic effects on murine bone marrow stromal (BMS) cells. To further analyze alterations to the cell, we evaluated the impact these VOCs have on membrane composition and hence fluidity. Both (*E*)-2-octenal and oct-1-en-3-ol exposure caused a shift to unsaturated fatty acids and lower cholesterol levels in the membrane. This indicates that the volatile organic compounds (VOCs) under investigation increased membrane fluidity. These vast changes to the cell membrane are known to contribute to the breakdown of normal cell function and possibly lead to death. Since bone marrow stromal cells are vital for the appropriate development and activation of immune cells, this study provides the foundation for understanding the mechanism at a cellular level for how mold exposure can lead to immune-related disease conditions.

Keywords

black mold; bone marrow stromal cells; membrane lipids; mold-associated volatiles; volatile organic compounds

INTRODUCTION

Fungi are ubiquitous and can be found naturally in air, water, soil and plants. Therefore, exposure to mold is fairly common. Molds can readily be transported from external sources inside of buildings through air circulation or can be carried by inhabitants of the building (Hossain, Ahmed et al. 2004). If the interior environment is conducive to growth, molds can flourish posing a hazard to those that occupy the building for long periods of time (Hodgson

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2000; Medicine 2004; Shoemaker and House 2006; Jaakkola and Jaakkola 2007; Joshi 2008). Sick building syndrome (SBS) is a loosely defined condition in which building occupants experience a series of acute symptoms related to adverse effects on health. These symptoms can include headache; eye, nose, or throat irritation; dry cough; dry or itchy skin; dizziness and nausea. In severe cases memory loss, pulmonary bleeding rheumatologic and other immune diseases have been reported (Hodgson 2000; Shoemaker and House 2006; Enriquez-Matas, Quirce et al. 2007; Jaakkola and Jaakkola 2007; Agency 2008; Joshi 2008; Straus 2009; WHO 2009). Of more recent concern, cases of infant idiopathic pulmonary hemorrhage have been linked to exposure to toxic molds such as *Stachybotryous chartarum* (Hossain, Ahmed et al. 2004). Evidence is clear that occupants of damp or moldy buildings are at heightened risk for developing respiratory infections including hypersensitivity pneumonitis and increased incidence of asthma and asthma-related symptoms (Rocha, Ansari et al. 2005; Enriquez-Matas, Quirce et al. 2007).

The toxic effects of molds have been linked to mycotoxins and volatile organic compounds (VOCs). VOCs are organic compounds that have a high vapor pressure at room temperature and some are associated with the musty smell of molds (Josephs, Derbyshire et al. 2004). Exposure to both mycotoxins and VOCs has been shown to induce multiple adverse effects including respiratory complications, immunotoxicity and carcinogenic effects in both animals and humans (Hodgson 2000; Kreja and Seidel 2002; Kreja and Seidel 2002; Korpi, Jarnberg et al. 2009; Inamdar, Masurekar et al. 2010; Inamdar, Moore et al. 2012; Inamdar, Zaman et al. 2012).

Epidemiological evidence strongly suggests a relationship between mold exposure and illness; however few studies have actually looked at the effects of VOCs on immune cell function. The competing immunosuppressive and immunostimulatory effects seen by various fungal compounds requires further investigation (Rocha, Ansari et al. 2005). We hypothesize that VOCs have deleterious effects on immune cell development and function, thereby compromising immune response and contributing to the observed negative health effects. Herein we show that two common VOCs (*E*)-2-octenal and oct-1-en-3-ol (Figure 1) have a cytotoxic effect on bone marrow stromal cells. In addition, we show that these volatiles cause alterations to the membrane composition of the cell. Bone marrow stromal cells are crucial for the development and activation of immune cells. Therefore, the observed toxic effects of the VOCs on this cell line can indirectly cause the immune system to break down or malfunction, which can lead to the deleterious health problems seen in individuals that have been exposed to toxic indoor molds (Kreja and Seidel 2002; Kreja and Seidel 2002; Korpi, Jarnberg et al. 2009; Inamdar, Masurekar et al. 2010; Inamdar, Moore et al. 2012; Inamdar, Zaman et al. 2012)

MATERIALS AND METHODS

Cell Culture

The murine bone marrow stromal (BMS) cell line was kindly provided by Dr. Salazar-Mather (Brown University, Providence RI). The cells were cultured under standard conditions in RPMI-1640 supplemented with 10% fetal bovine serum and 1% penicillin/

streptomycin and incubated at 37°C with 10% CO₂. The cells were harvested, counted, and split at 90% confluence.

3-(4,5-dimethylthiazol-2-yl) 2,5-diphenyl tetrazolium bromide (MTT) Dye Cytotoxicity Assay

BMS cells were placed in 96-well plates in triplicate at 10⁴ cells per well in RPMI medium. Cells were allowed to adhere for 24 h, followed by replacement of the RPMI media containing oct-1-en-3-ol or (*E*)-2-octenal in RPMI ranging from 0.01% - 0.1% (100 ppm to 1000 ppm) for various periods of time. Cytotoxicity was determined using an MTT assay kit (Trevigen) following manufacturer's protocol. Briefly, 25 µL of MTT dye was added to each well of VOC-treated and wells containing media alone, and incubated for 3 hours. Following incubation 100 µL of sodium dodecyl sulfate (SDS) containing detergent was added and incubated for 1 hour at room temperature. Treated plates were analyzed at 570 nm using a Spectramax 190 microtiter plate reader spectrophotometer. Data was analyzed using SoftMax Pro (Molecular Devices). Absorbance values were converted to percent cell survival by setting control (untreated) samples to 100% survival. Experiments were performed in technical triplicate and biological duplicate and the data pooled and averaged.

Lipid Analysis

BMS cells were placed into a single well of a 6-well flask at 10⁶ cells per well. The cells were exposed to 0.05% (500ppm) of oct-1-en-3-ol (corresponding to 70-80% survival) or (*E*)-2-octenal for 25 minutes. Cells were removed with trypsin and washed extensively with PBS buffer. Lipid extraction was performed using the method described by Bligh and Dyer (Bligh and Dyer 1959). Briefly, cells were extracted with 0.5:1:0.4 (CHCl₃: MeOH: H₂O) and cell debris removed by centrifugation. The extract was then converted to a 2 phase Bligh-Dyer of final composition 1:1:0.9 (CHCl₃:MeOH:H₂O) and gently centrifuged to separate phases. The organic layer was retained and evaporated to dryness under a stream of N₂. Lipids were analyzed as fatty acid methyl esters (FAME) as previously described (Ichihara and Fukubayashi 2010). Analysis was performed on an Agilent 7890A GC coupled to an Agilent 5975C mass spectrometer equipped with a Agilent 190913-433 column (30 m x 250mm x 0.25 mm). The oven was programmed as follows: 80°C (hold for 1 min); 160°C at 20°C/min (hold for 5 min); 198°C at 1°C/min (hold for 1 min); 250°C at 5°C/min (hold for 5 min); 280°C at 20°C/min (hold for 5 min). Samples were run in technical and biological duplicate. A two tailed χ^2 analysis of the data was performed to confirm that differences observed in VOC treated cells were dependent on the presence of VOC.

Cholesterol Assay

BMS cells were grown in the presence or absence of (*E*)-2-octenal or oct-1-en-3-ol at 0.05% (500 ppm) for 25 minutes. Cellular lipids were extracted as described above for lipid analysis. Cholesterol content in untreated and treated cells was analyzed using a cholesterol determination kit (Wako Chemicals). Briefly, lipid extracts were diluted with 1 mL of distilled water and 100 µL of each diluted sample was used to perform the assay. Cholesterol content was analyzed at 600 nm and 700 nm on a Spectramax 190 micro titer plate reader spectrophotometer using Softmax Pro software for analysis. Cholesterol concentration was calculated according to manufacturer's suggestions.

RESULTS

Fungal volatiles are cytotoxic to murine bone marrow stromal cells

BMS cells were exposed to the fungal volatiles oct-1-en-3-ol or (*E*)-2-octenal for times ranging from 0 to 30 minutes (Figure 2). These two volatiles were chosen based on their prevalence in toxic mold species and the observed defects in dopaminergic neurons in *Drosophila* (Inamdar, Masurekar et al. 2010). In addition, these two particular volatiles were found to be dominant in a study looking at the prevalence of microbial volatiles in United States homes (Ryan and Beaucham 2013). Cell survival following exposure was measured by MTT analysis. Cells that were exposed to both oct-1-en-3-ol and trans-2-octenal died following incubation with the volatiles with the greatest effects seen at 0.05% (Figure 2). (*E*)-2-octenal exposure resulted in a more rapid and profound cell death rate as compared to that which was observed in cells exposed to oct-1-en-3-ol. By 30 minutes post exposure approximately 50% cell survival was observed at the highest concentration for oct-1-en-3-ol, whereas with (*E*)-2-octenal the highest concentration at 30 minutes resulted in about 25% cell survival (Figure 2). These results confirmed that fungal volatiles have a toxic effect on murine bone marrow stromal cells at fairly low concentrations and following a short time of exposure.

Cells exposed to fungal volatiles demonstrate changes to their cellular membranes

In order to further analyze cellular changes caused by exposure to fungal volatiles, cells were treated with either oct-1-en-3-ol or (*E*)-2-octenal for 25 minutes at 0.05% final concentration. This dose and time were chosen for both volatiles as this was when cells exhibited a 70-80% survival rate and would be indicative of early cellular modifications that would possibly contribute to cell death. This dosage is also similar to a study investigating the effects of oct-1-en-3-ol on human embryonic cells (Inamdar, Moore et al. 2012). FAME analysis of cells treated with either oct-1-en-3-ol or (*E*)-2-octenal for the time and dose indicated above are presented in Table 1. GC-MS analysis revealed a significant decrease in unsaturated fatty acids as compared to the control samples, indicating that these VOCs perturb membrane fluidity which can lead to cellular damage. Alterations in membrane fluidity were further confirmed upon analysis of total membrane cholesterol content. Upon exposure to oct-1-en-3-ol and (*E*)-2-octenal cells displayed significant decreases in cholesterol content of the cell membrane. Control cells had on average 14.5 mg dl⁻¹ whereas cells treated with oct-1-en-3-ol and (*E*)-2-octenal had 1.95 mg dl⁻¹ and 9.7 mg dl⁻¹ respectively (Table 1). This would indicate an increase in membrane fluidity for those cells exposed to the VOCs.

DISCUSSION

The relationship between indoor exposure to toxic mold and human health is only beginning to be understood. While evidence exists of strong correlations between exposure and infectious and allergic diseases, the effects on immune cell function is poorly understood. The data presented here contributes to the understanding of the observed relationships between immunity and mold exposure by analyzing immune cell death as a result of fungal volatile exposure. Enhanced cytotoxicity has been observed in human embryonic stem cells

exposed to oct-1-en-3-ol at comparable levels shown here (Inamdar, Moore et al. 2012) as well as neurotoxicity seen in *Drosophila melanogaster* following exposure to microbial volatile organic compounds (Inamdar, Masurekar et al. 2010). Exposure to oct-1-en-3-ol has been linked to increased allergy presence in single family homes (Araki, Kanazawa et al. 2012) indicating a role for microbial volatile organic compounds and human health. Based on prior evidence of cytotoxicity in several cell types along with observed shifts in TH1 and TH2 immune responses leading to enhanced allergy and hypersensitivity responses, we proposed that bone marrow stromal cells, which provide the necessary support network for the development of appropriate immune responses could also be affected by VOC exposure, leading to alterations in immune cell development and ultimately to decreased TH1 and increased TH2 responses as seen in individuals chronically exposed to indoor mold. Therefore we chose to examine the effects of two known fungal volatiles, oct-1-en-3-ol and (*E*)-2-octenal on murine bone marrow cells as a baseline measure of immune function following exposure. Both volatiles were cytotoxic in nature at relatively low concentrations and in a short amount of time. We then investigated the changes in the cell structure that could contribute to the cell death observed in treated cells. The lipid profile of cells that were exposed to the volatiles as compared to the control cells was significantly different. The degree of lipid saturation was greatly modified in treated cells as compared to the control, indicating again changes to membrane content that could lead to the initiation of cell death pathways. Finally, the cholesterol content is severely altered in exposed cells as compared to that seen in untreated control cells. Cholesterol is a predominant component of the plasma membrane of all mammalian cells and plays a major role in determining membrane function. Cholesterol content alters membrane fluidity, which has downstream effects such as augmented ion transport and membrane protein function (Cooper, 2004; Gleason, Medow et al. 1991). Typically, the higher the membrane cholesterol content, the lower the membrane fluidity. It has been shown with other toxins such as ethanol, that membrane fluidity plays a key role in toxin induced cell stress (Sergent, Pereira et al. 2005). Additionally, decreases in cholesterol content are indicative of a reduction of lipid rafts in the membrane and has been linked to ligand-independent activation of Fas and apoptosis (Gniadecki 2004). Taken together, these membrane modifications can contribute to the cell death observed in BMS cells exposed to VOCs secreted by toxic black mold species. As these effects are seen in immune-supporting cells, we can suggest a mechanistic link between volatile-induced cell death and immunosuppressive events that can directly contribute to the cause of ill health in individuals that are exposed to indoor molds for extensive periods of time. Alternatively, the observed stromal cell death could lead to misregulation of immune cell development and could contribute to heightened immune responses like hypersensitivity or allergy that is commonly seen in mold-exposed individuals. These studies provide a platform for further investigation of individual immune cell function and further analysis of intracellular signalling that is induced following exposure that can lead to cell death in bone marrow stromal cells and ultimately decreased or altered immune activity.

Acknowledgements

This work was supported by an Institutional Development Award (IDeA) from the National Institute of General Medical Sciences of the National Institutes of Health under grant number 8 P20 GM103430-12.

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Figure 1.
Structures of mold VOCs (*E*)-2-octenal (**1**) and oct-1-en-3-ol (**2**).

Figure 2.

Effect of oct-1-en-3-ol and (*E*)-2-octenal on murine bone marrow stromal cells. Cultured cells were either unexposed (control) or exposed to (A) oct-1-en-3-ol or (B) (*E*)-2-octenal for times indicated from 0-30 minutes at concentrations ranging from 0.01% to 0.1%. Cell survival was measured by MTT analysis. Data shown represents the mean $\pm$ SE of at least two independent experiments.

Table 1

Analysis of membrane composition of Bone marrow stromal cells in the presence/absence of VOC

Sample	% saturated fatty acid	% unsaturated fatty acid	Cholesterol (mg dL ⁻¹)
Control	68.54 ± 11.21 ^a	26.05 ± 6.01	14.25 ± 0.64
Oct-1-en-3-ol	83.75 ± 16.04	5.34 ± 6.18	8.75 ± 0.78
(<i>E</i>)-2-octenal	100	n.d. ^b	2.25 ± 0.21

^a standard deviation^b not detected