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Differential cytotoxicity of long-chain bases for human oral gingival epithelial keratinocytes, oral fibroblasts, and dendritic cells

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HIGHLIGHTS

- Long-chain bases sphingosine and dihydrosphingosine are in saliva and have antimicrobial activity against oral pathogens.
- We determined the toxicity of sphingosine, dihydrosphingosine, and phytosphingosine for GE keratinocytes, GF, DC, and SCC cells.
- The LD_{50} of long-chain bases for GE keratinocytes, GF, SCC cells, and DC were considerably higher than their MIC for oral pathogens.
- This finding is important in pursuing the future potential of long-chain bases in treating periodontal and oral infections.

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ABSTRACT

Long-chain bases are present in the oral cavity. Previously we determined that sphingosine, dihydrosphingosine, and phytosphingosine have potent antimicrobial activity against oral pathogens. Here, we determined the cytotoxicities of long-chain bases for oral cells, an important step in considering their potential as antimicrobial agents for oral infections. This information would clearly help in establishing prophylactic or therapeutic doses. To assess this, human oral gingival epithelial (GE) keratinocytes, oral gingival fibroblasts (GF), and dendritic cells (DC) were exposed to 10.0–640.0 μM long-chain bases and glycerol monolaurate (GML). The effects of long-chain bases on cell metabolism (conversion of resazurin to resorufin), membrane permeability (uptake of propidium iodide or SYTOX-Green), release of cellular contents (LDH), and cell morphology (confocal microscopy) were all determined. GE keratinocytes were more resistant to long-chain bases as compared to GF and DC, which were more susceptible. For DC, 0.2–10.0 μM long-chain bases and GML were not cytotoxic; 40.0–80.0 μM long-chain bases, but not GML, were cytotoxic; and 80.0 μM long-chain bases induced cellular damage and death in less than 20 min. The LD_{50} of long-chain bases for GE keratinocytes, GF, and DC were considerably higher than their minimal inhibitory concentrations for oral pathogens, a finding important to pursuing their future potential in treating periodontal and oral infections.

1. Introduction

Saliva contains neutral lipids; cholesterol; mono-, di-, and triglycerides; free fatty acids; wax esters; cholesterol esters; squalene; and long-chain sphingoid bases (Brasser et al., 2010, 2011a,b; Defago et al., 2011; Kensche et al., 2013; Larsson et al., 1996; Palmerini et al., 2011). Many of these lipids have innate

Abbreviations: GE, human oral gingival epithelial keratinocytes; GF, oral gingival fibroblasts; DC, dendritic cells; GML, glycerol monolaurate; SCC, oral squamous cell carcinoma cells; LDH, lactate dehydrogenase; MFI, median fluorescence intensity; MIC, minimal inhibitory concentrations.

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immune functions: they are antimicrobial, influence the interaction of oral microorganisms with the salivary pellicle, impede microbial adherence to oral surfaces, and create a hydrophobic layer protecting teeth from demineralization (Bibel et al., 1992; Kensche et al., 2013).

The long-chain bases sphingosine, dihydrosphingosine, and phytosphingosine have variable antimicrobial activity against a variety of Gram-positive and Gram-negative bacteria including *Escherichia coli*,*Staphylococcus aureus*, and *Corynebacterium species* (Fischer et al., 2012, 2013) and more potent antimicrobial activity against oral bacteria including *Streptococcus sanguinis*, *Streptococcus mitis*, *Fusobacterium nucleatum*, and *Porphyromonas gingivalis* (Fischer et al., 2012, 2013). For oral bacteria, mean minimal inhibitory concentrations (MIC) range from 0.1 to 2.5 μM (e.g., 0.3–7.8 μg/ml) with the individual MIC dependent upon the specific long-chain base and oral microorganism tested.

Long-chain bases are present in the oral cavity at 1.6–16.6 μM (e.g., 0.5–4.9 μg/ml) concentrations (Brasser et al., 2011a). However, little is known about their cytotoxicities for oral cells at various concentrations, an important step in considering their potential as therapeutics for preventing or treating oral infections. In this study, we determined the cytotoxicities and lethal dose 50 (LD_{50}) values of long-chain bases for human oral gingival epithelial (GE) keratinocytes, oral gingival fibroblasts (GF), and dendritic cells (DC). The lipid glycerol monolaurate (GML) was used as a negative control. We also included oral squamous cell carcinoma (SCC) cells as controls, which are known to be susceptible to the cytotoxic effects of long-chain bases and their derivatives (Shirahama et al., 1997b).

2. Material and methods

2.1. Solutions, media, and long-chain bases

0.01 M sodium phosphate with 0.14 M NaCl, pH 7.2 (PBS) was used as a diluent and as a control solution. Serum-free lymphocyte growth medium 3 (LGM-3, Lonza Walkersville, Inc., Walkersville, MD) was used to cultivate GE keratinocytes, GF, and DC. Sphingosine (D-sphingosine), dihydrosphingosine (D-erythro-dihydrosphingosine), and phytosphingosine were obtained from Sigma–Aldrich (St Louis, MO). GML was obtained from LKT Laboratories (St. Paul, MN). GML is non-toxic for human and murine cells (Peterson and Schlievert, 2006). Long-chain bases were dissolved in a chloroform:methanol solution (2:1) and their purities were confirmed by thin-layer chromatography. Chloroform:methanol solutions were dispensed in glass tubes; dried under nitrogen; and resuspended and diluted in PBS to 640.0 μM stock solutions.

2.2. Cell culture

Primary, first passage GE keratinocyte cell lines GE363, GE367, GE368, GE369, GE370, and GE371, prepared in a previous study and stored in liquid nitrogen were used in this study (Joly et al., 2005). These cells were from healthy gingival tissue samples obtained from healthy non-smoking individuals who underwent crown lengthening or canine exposure procedures. Informed consent was obtained from these individuals per a reviewed and approved protocol from the University of Iowa Institutional Review Board for the use of human subjects in research. Concentrations of GE keratinocytes were determined and adjusted to contain 1.0×10^5 viable cells/ml LGM-3.

Oral fibroblast primary cell lots GF365, GF367, GF368, and GF369 were isolated from the connective tissue separated from the epithelium in the above procedure. Briefly, isolated connective tissue was cut into small, 2–4 mm pieces and allowed to attach to a 60 mm tissue culture plate and covered with DMEM/10% FBS with antibiotics. The connective tissue was mixed with trypsin (225.0 USP units/mg) at 37 °C, and then incubated in modified FAD media. Cells were pelleted by centrifugation for 10 min at $30 \times g$ (IEC HN-SII, International Equipment Company, Needham Heights, MA) and suspended in modified FAD media and mixed. Cells were counted and added to six-well plates (Corning, NY) at a density of 2.5×10^5 cells/well in DMEM/10% FBS with antibiotics. Concentrations of GF were determined and adjusted to contain 1.0×10^5 viable cells/ml LGM-3.

Primary human myeloid DC were used (StemCell Technologies, Inc., Vancouver, BC Canada). Concentrations of DC were determined and adjusted to contain 1.0×10^5 viable cells/ml LGM-3.

University of Michigan squamous cell carcinoma (UM-SCC) cell lines SSC-15, SCC-19, SCC-84, SCC-99, and SCC-1483 were used (Brenner et al., 2010). These cell lines were established from head and neck cancer patients who gave written informed consent to participate in studies reviewed and approved by the University of Michigan Medical School Institutional Review Board. Concentrations of UM-SCC were determined and adjusted to contain 1.0×10^5 viable cell/ml LGM-3.

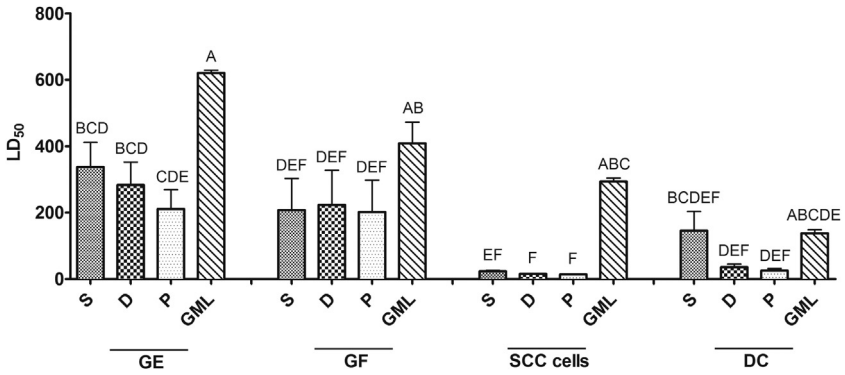

Fig. 1. Long-chain bases affect cell metabolism (conversion of resazurin to resorufin) and there are differing mean lethal dose 50 (LD_{50}) values of sphingosine (S), dihydrosphingosine (D), phytosphingosine (P), and glycerol monolaurate (GML) for oral gingival epithelial (GE) keratinocytes, oral fibroblasts (GF), dendritic cells (DC), and oral squamous cell carcinoma (SCC) cells. The effects of long-chain bases on cell metabolism (conversion of resazurin to resorufin) were first determined. Percent cytotoxicity was then defined as the median fluorescence intensity (MFI) of resazurin in cell culture media of cells treated with dilutions of long-chain bases/MFI of resazurin in cell culture media of untreated cells × 100, and the LD_{50} values were determined from the dose response curve where the 50% cytotoxicity intercepts with the long-chain base concentration on the *x*-axis. Values not connected by the same letter are significantly different. The statistical analysis of the data in this graph can be found in Table 1.