Variability in lipids isolated from human cheek cells

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Major lipid classes were examined in human cheek cell preparations isolated 3 or 7 days apart. In 13 of 14 subjects, samples isolated 7 days apart had less phospholipid and a higher ratio of sterol + nonpolar lipid to phospholipid than samples isolated 3 days apart. It was concluded that frequency of cheek cell collection is one factor that contributes to variability noted in studies of human cheek cell lipids.

Keywords: buccal cells; cheek cells; maturation; lipid classes; phospholipids

Introduction

Analysis of human cheek cell lipids has been suggested as a non-invasive method for monitoring dietary lipid intake.[1,2] Nevertheless, except for studies on fatty acid composition,[1-3] data on the lipids present in human cheek cells are virtually nonexistent. To our knowledge, the only available data on lipid classes present in buccal epithelial cells are for tissue isolated from pigs.[4,5]

In our studies with human cheek cell lipids,[3] we observed considerable variability in cheek cell components. In view of the reported[6] turnover time of 5 days for buccal epithelial cells, we wondered whether the frequency of cell collection could influence the quantitative nature of the resulting cheek cell lipid data. In the present study we examined the major lipid classes present in human cheek cells so that the lipids in cheek cells isolated 3 days apart could be quantitatively compared with those isolated 7 days apart.

Methods and materials

Subjects

Cheek cells were isolated over a period of 7 weeks from 14 subjects (six males and eight females, ages 22–50, on free-living diets). Subjects were required to refrain from eating, brushing their teeth, or using mouthwash, lipstick, etc. on the morning that the cheek cells were collected. After obtaining the first cheek cell sample, which was not used in the study, successive cheek cell samples were isolated so that there was a 3 day interval (3 day samples) or a 7 day interval (7 day samples) between check cell collections. Both 3-day and 7-day samples were collected from all subjects. Thus, four 3-day samples and four 7-day samples were collected from each subject.

Isolation of cheek cells

Cheek cell samples were collected essentially as described previously[3] except for the number of times each cheek surface was scraped. Prior to scraping, subjects rinsed their mouths two times with distilled water, swishing vigorously and discarding the rinses. After the second rinse was discarded, cells were collected by gently scraping the inside of one cheek from the top to the middle with eight strokes of a plastic spoon and rinsing the cells from the spoon into a collection cup. This was followed by gently scraping the same cheek from the bottom to the middle with eight strokes of the spoon and rinsing the residue from the spoon into the collection cup and then gently scraping the same cheek using five strokes of the spoon from the top to the middle, followed by five strokes from the bottom to the middle and rinsing the cell residue from the spoon into the collection cup. These three steps were repeated for the other cheek. After both cheeks were scraped, the subject used distilled water to rinse any loosened cheek cells into the collection cup. Gentamycin sulfate (250 μg; Sigma, St. Louis, MO USA) was added to the collected contents and the cells were pelleted by centrifugation as described previously.[3]

The centrifuge tube was sealed with parafilm and the pellet was frozen at 4° C for 2 hr. The hardened pellet was transferred into a 5 mL (0.004–0.006 in clearance) Potter-Elvejhem tissue grinder (VWR Scientific, Bridgeport, NJ USA) with the aid of a spatula and a minimal amount of distilled water, resuspended using 10 passes of a hand-driven pestle, and diluted to 2 mL with distilled water. A 20 μL aliquot of this suspension was used for cell count and 1.8 mL of the suspension was extracted and analyzed for lipid classes.

The 20 μL portion of the resuspended pellet was diluted

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with 20 μL of Coomassie Blue (500 mg in 50 mL of a 50% (vol/vol) ethanol solution) to stain cells for counting by light microscopy. A 10 μL aliquot of the stained cell suspension was pipetted into each of two wells in a hemacytometer, and the total cells in the nine primary squares of each well were counted. The two estimates were averaged and expressed as number of cells per total pellet.

Lipid extraction and thin layer chromatography

Total lipids were extracted from 1.8 mL of the original cell suspension as described previously[3] and stored in 0.5 mL of chloroform-methanol (95/5, vol/vol) at 4° C until analyzed by thin layer chromatography (TLC). Whatman Linear K Silica Gel, LK 5D plates (American Scientific, McGaw Park, IL USA), containing 19 prescored channels and a preadsorbant area, were used for TLC. Samples and standards were spotted on even-numbered lanes just below the silica gel-preadsorbant interface. Sample aliquots consisting of 30, 60, and 100 μL each were spotted on three lanes and 10 μL aliquots of standard mixtures in increasing concentrations were spotted on four of the adjacent channels on each TLC plate. The lipids were moved out of the preadsorbant layer by briefly placing the spotted plates in the appropriate solvent system (see below) until the solvent front was about 0.5 cm above the preadsorbant area. This was done three times for each plate and included an air-drying interval in a hood after each exposure to solvent.

All samples were spotted on at least two TLC plates, one to resolve nonpolar lipids and the other to resolve polar lipids. To resolve polar lipids, the TLC plates were developed to a height of 12 cm in a polar solvent system, consisting of chloroform/ethanol/water/triethylamine (30/34/2/35; vol/vol/vol/vol), allowed to air dry in a hood and then developed to a height of 19 cm in the nonpolar solvent system, consisting of hexane/diethyl ether/formic acid (85/15/1; vol/vol/vol). The nonpolar lipids were resolved by developing the plates to a height of 19 cm in the nonpolar solvent system.

Quantitative analysis of lipid classes

To aid in quantifying the major lipid classes present in cheek cells, ten μL aliquots of standard mixtures in increasing concentrations were spotted on four channels of each TLC plate. The standard mixtures were prepared from individual lipids of greater than 98% purity purchased from Sigma Chemical Co. Mixtures of sphingomyelin, phosphatidylcholine, phosphatidylethanolamine, cholesterol, and cholesterol sulfate were used to help quantify the major polar lipid classes, and standard mixtures containing cholesterol, free fatty acid, triglyceride and cholesterol ester were used to help quantify the major non polar lipids.

After development, the TLC plates were allowed to air dry completely in a hood. The air-dried plates were immersed in a solution of 0.1% $CuSO_4$ in 8% H_3PO_4[7] for 20 seconds and placed in a hood for 30 min to permit the excess moisture to evaporate. Visualization was accomplished by heating the plates in a 170° C oven for 5 min to char the nonpolar lipids or for 10 min to char the polar lipids. Plates were stored overnight at room temperature in the dark prior to quantitative analysis, which was conducted using a Kratos/Schoffel Model SD-3000 Spectrophotodensitometer (Westwood, NJ USA) equipped with a Hewlett Packard 3390A Reporting Integrator (Avondale, PA USA). Standard curves were generated from the standard lipid mixtures present on each plate and used to quanitfy the major lipid classes present in the cheek cell samples as μg of polar or nonpolar lipid/10^6 cells. The ratio of nonpolar to polar lipid was also reported.

Identification of lipid classes

Comparisons of unknowns and standards spotted on the same TLC plate, as well as the relative retention ratios of lipid classes in solvent systems reported in the literature,[8] were used to identify lipid classes. In addition, spray reagents, including molybdenum blue, chloroxbenzidine, and orcinol, prepared as described by Skipski and Barclay,[9] were employed to aid in identification of phospholipids, sphingolipids, and glycolipids, respectively.

Results and discussion

As has been noted previously,[3] the cell populations isolated were heterogeneous, consisting mainly of single cells with the occasional presence of sheets of cells. In most samples a small number of cells were broken open and had a bubbly or fuzzy appearance. However, the proportion of cells that appeared to be obviously broken was small and relatively constant. It was not clear whether the broken cells resulted from the action of oral microorganisms prior to cell collection or to the procedure used to resuspend the cell pellet. Nevertheless, for most samples there was no direct evidence for the presence of microorganisms or of residual food particles in the cell suspensions. Thorough rinsing of the mouth prior to cell collection may be important in this regard. Although some cheek cells were undoubtedly discarded in the initial rinses, we observed that the majority of the cheek cell sample was collected in the final rinse. Thus it appears that if the initial mouth rinses are thorough, the scraping procedure acts primarily to loosen cells, and the subsequent vigorous rinsing releases the loosened cells.

Several lipid classes were detected in cheek cell extracts. Major lipids observed were sphingomyelin, phosphatidylcholine, phosphatidylethanolamine, cholesterol-3-sulfate, cholesterol ester, cholesterol, free fatty acid, and triacylglycerols. Minor lipids included phosphatidylserine, phosphatidylinositol, monoacylglycerol, and diacylglycerol. In addition, based on TLC relative retention ratios and specific color reactions, cheek cell lipids contained at least two species of ceramides and several glycolipids, which included cerebrosides, sulfatides, and glycosylceramide. All of these lipids are common to epidermal tissues[4,5,10–12] and many, including the non-acylated glycosylceramides, have been identified[4,5] in the buccal epithelium isolated from pigs.

The percentages of the major lipids detected in human cheek cells isolated 3 or 7 days apart are given in *Table 1*. There was a trend for phospholipids to decrease and for some of the nonpolar lipids to increase in the 7 day samples. However, the differences were not statistically significant as there was considerable sample-to-sample variation. On an absolute basis, the overall value as the mean ± S.D. for the total phospholipid fraction was only 115 ± 35 μg/10^6 cells compared with 858 ± 250 μg/10^6 cells for the nonpolar + sterol lipids.

Table 1 Major lipid classes in human buccal cells isolated 3 or 7 days apart

Class	3 Day	7 Day
Sphingomyelin	2 (0.7)	2 (0.7)
Phosphatidylcholine	8 (2)	6 (2)
Phosphatidylethanolamine	4 (2)	3 (1)
Σ phospholipids	14 (2)	11 (2)
Cholesterol Sulfate	7 (4)	6 (4)
Cholesterol	33 (7)	27 (5)
Free Fatty Acid	14 (6)	23 (7)
Triacylglycerol	18 (6)	20 (7)
Cholesterol Ester	14 (5)	14 (3)
Σ sterol + nonpolar lipids	86 (2)	90 (2)

Values are the weight percent of the total quantified lipids as determined by photodensitometry and represent the mean (and standard deviation) of 56 samples; 4 from each of 14 subjects.

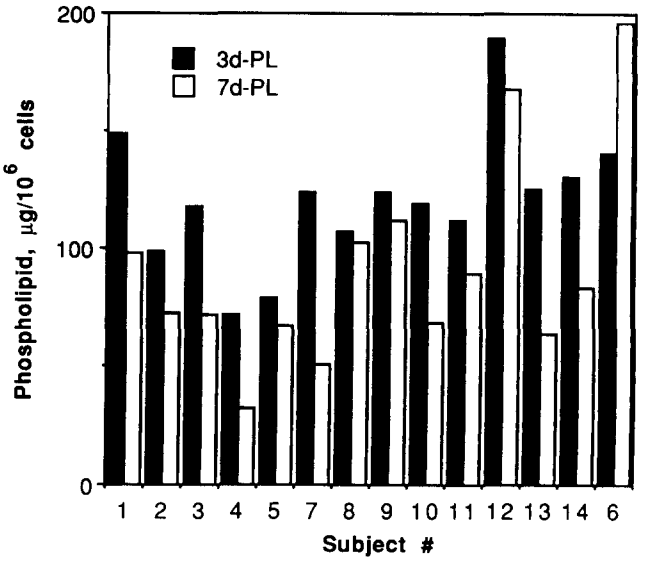

Figure 1 Total phospholipid (μg/10^6 cells) in human cheek cells isolated 3 or 7 days apart from 14 subjects on a free-living diet. Each bar represents the average of four samples.

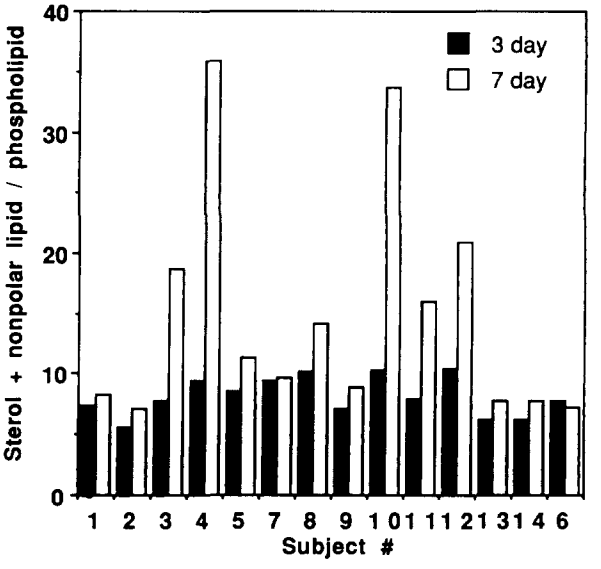

Figure 2 Ratio of sterol + nonpolar lipid to phospholipid in human cheek cells isolated 3 or 7 days apart from 14 subjects on a free-living diet. Each bar represents the average of four samples.

The relatively high nonpolar lipids relative to phospholipids in human cheek cell preparations and the high variability associated with cheek cell values has been noted previously.[3]

The mean weight percentage ± S.D. for total phospholipid in human cheek cells (13 ± 2) observed in our studies was much lower than that reported by Wertz et al.[4] for lipids in the buccal epithelia isolated from pigs (38.2 ± 3.7). It is possible that the superficial layers of human and pig epithelia simply contain different proportions of lipid classes, but other potential explanations for the observed differences should be considered. Epithelial tissues consist of layers of cells ranging from the innermost basal layer to the surface squamous layer; however, characteristic strata, as are seen in keratinized epithelia,[5] are less well defined[5,13] in buccal epithelia. If cheek cells can undergo a maturation pattern similar to that of epidermal cells, the procedure used to isolate cheek cells would yield primarily the most easily sloughed, outermost surface of cells, which in skin[10] contain extremely low proportions of phospholipid. In contrast, the procedure used by Wertz et al.[4] would result in total lipid from full thickness buccal epithelium. Nevertheless, the above explanation is at odds with the recent work of Squire, Wertz, and Cox,[5] who observed that serial sections of pig buccal epithelium lacked the dramatic differences in phospholipid and nonpolar lipid classes observed in keratinized tissue. In that study of pig buccal lipids,[5] even the innermost layer of cells consisted of about equal percentages of phospholipid and nonpolar lipid. Although species differences may contribute to the apparently disparate findings, other factors could be involved. These include the possibility that in human studies polar lipids from the outermost desquamating cells were preferentially lost into saliva, the possibility that the scraping procedure used in the present study released a relatively specific population of cells, and the possibility that our experimental design, which involved repeated scrapings, induced buccal cells to adopt a pattern of maturation characteristic of keratinized tissue.

We also compared the 3 and 7 day phospholipid values for each individual. The phospholipid values as μg/10^6 cells are summarized in *Figure 1* and the ratio of sterol + nonpolar lipids to phospholipids are presented in *Figure 2*. In 13 of the 14 subjects, the phospholipids were decreased and the ratio of sterol + nonpolar lipids to phospholipids was increased in the samples collected 7 days apart. The high inter-individual variation necessitated the use of Wilcoxon's non-parametric rank test.[14] Using this test, both the decrease in phospholipids and the increase in the ratio of the sterol + nonpolar lipids to phospholipids were statistically significant ($P < 0.005$).

Although, as discussed above, caution should be exercised in drawing conclusions, these results are consis-

tent with the concept that human buccal cells may undergo a maturation, involving alterations in lipid content, which could be analogous to the degradation of subcellular structure and membrane loss that is known to occur in keratinized epithelial cells as they mature.[10,11] Regardless of the reason for the differences observed, frequency of cheek cell collection appears to be a factor contributing to the variability in lipid classes isolated from human cheek cell preparations.

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