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Branched-chain fatty acids, increased in tears of blepharitis patients, are not toxic for conjunctival cells

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Competing Interest: None declared.

ABSTRACT

Purpose: The composition of the meibum of blepharitis patients is characterised by increased levels of branched-chain fatty acids (BCFAs) that return to normal values in patients treated with cyclins and lid hygiene. The aim of this study was to determine if BCFAs had toxic effects on conjunctival cells related to the disease.

Methods: Chang and IOBA-NHC conjunctival human cells were treated with BCFAs (isoC16, and isoC20) or palmitic acid as a control for 4 h or 24 h at 50 μ M or 100 μ M. Morphological and functional changes were investigated by measuring mitochondrial dehydrogenase activity, cell permeability, mitochondrial depolarisation, chromatin condensation, IL-1 β and reactive oxygen species production.

Results: None of the fatty acids modified the parameters of cytotoxicity in conjunctival cells in Chang or IOBA-NHC cell lines. Only the mitochondrial dehydrogenase activity was significantly decreased in relation to the isoC20 concentration increase.

Conclusions: The increase in BCFAs in the tears of blepharitis patients does not consistently participate in the conjunctival cell changes throughout the course of the disease. Instead, it is likely an adaptive response of the ocular surface to the lack of tears, possibly increasing meibum fluidity, thus enhancing lacrimal film stability.

INTRODUCTION

Blepharitis is a very frequent cause for consultation in ophthalmology [1] because of the procession of symptoms that are generated. [2] In previous papers, we showed that posterior blepharitis associated with meibomian gland dysfunction (MGD) leads to changes in meibomian lipids, particularly to a significant decrease in saturated fatty acids and a significant increase in the proportion of branched-chain fatty acids (BCFAs) [3], which lowered after minocycline treatment and lid hygiene. [4] These modifications should involve changes in the quality and therefore in the function of the lipidic phase of the tear film, whose most important role is to avoid evaporation of tears from the ocular surface. [5] Since the tear film is in permanent contact with conjunctival cells, we aimed to determine if an increase in BCFAs was toxic for conjunctival cells or if instead it was an adaptive response of the ocular surface to the lack of tears. On one hand, BCFAs may have toxic effects on conjunctival cells, as other BCFAs such as phytanic acid and methyltetradecanoic acid affect astrocytes, smooth muscle cells and breast cancer cells. [6, 7] On the other hand, as observed in vitro [8], BCFAs may improve tear stability, counterbalancing the lack of tears in blepharitis patients by facilitating the spread of the tear film on the ocular surface.

To clarify this point, we evaluated the cytotoxicity of BCFAs on cultured conjunctival cells by assessing mitochondrial dysfunction, inflammation and apoptosis, comparing them with palmitic acid (C16), a straight-chain fatty acid used as a control. We studied the effect of two of the main BCFAs present in tear lipids that were significantly increased in blepharitis: isoC16 and isoC20. [3] We compared the results in two cell lines: the Wong-Kilbourne derivative of Chang conjunctival cell lines, which has been widely used for toxicological in vitro studies, and the IOBA-NHC cell line, which showed a toxicological profile comparable to the Chang cells [9], to lessen the impact of the presence of HeLa marker chromosomes in the Chang cells.

MATERIALS AND METHODS

Cells

Two conjunctival cell lines of human origin were cultured in standard conditions (5% CO₂, 95% O₂, 37°C). Chang cells (Wong-Kilbourne derivative of Chang conjunctiva, clone 1-5c-4, ATCC CCL-7) were cultured in Eagle's minimal essential medium (DMEM) with a high glucose concentration (4.5 g/L) (Gibco™, Invitrogen, Bioblock, Illkirch, France) supplemented with 10% fetal calf serum (FCS) (Eurobio, Courtaboeuf, France), 25 mM HEPES (Gibco™), 20 µg/mL gentamicine (Gibco™). A second human conjunctival cell line spontaneously arising from a primary culture of human conjunctival epithelium (IOBA-NHC) was cultured in DMEM/F-12 (1:1) (Gibco™) supplemented with 2 ng/mL epidermal growth factor (EGF) (AbCys, Paris, France), 1 µg/mL bovine pancreas insulin (Sigma-Aldrich, Saint Quentin Fallavier, France), 0.1 µg/mL cholera toxin (Sigma-Aldrich), 5 µg/mL hydrocortisone (Sigma-Aldrich), 10% FCS, and 20 µg/mL gentamicin (Gibco™). Cells from passages 9 and 10 (following ATCC initial passage 65 or following the provision of IOBA-NHC cells) were used in all experiments. The medium was changed every 2–3 days, and cell growth was assessed daily by phase-contrast microscopy. Cells were seeded at a density of 10⁵ cells/mm² in various supports, depending on the assay (see below).

Cell treatment

Stock solutions of isoC16, isoC20 and C16 (Sigma-Aldrich) were prepared at a concentration of 20 mM in dimethylsulphoxide (DMSO) hybrimax solutions (Sigma-Aldrich). Cells were incubated with 50 or 100 µM of fatty acids for 4 h or 24 h (DMSO, 1% final concentration). Control cells were incubated with 1% DMSO alone. Experiments were done at least in triplicate.

Measurement of cell viability

Cell viability was assessed using the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) assay for mitochondrial succinate dehydrogenase (SDH) activity, as we have already described. [10]

Measurement of cell growth

Cell growth was estimated through the protein content, which was determined in 24-well plates by a fluorescamine assay, as we have previously described. [10]

Measurement of cell permeability

Cell permeability was determined after staining with the phenanthrene dye propidium iodide (excitation $\lambda=540$ nm, emission $\lambda=625$ nm) (Sigma-Aldrich), which enters dead cells only. [11] Propidium iodide was used at a final concentration of 5 $\mu\text{g/mL}$ on a cell suspension adjusted to 10^6 cells/mL. Fluorescence was immediately quantified using flow cytometry in 10,000 cells on a 4-decade logarithmic scale of fluorescence on a FACScan flow cytometer (Becton Dickinson, UK) at 488-nm and 590-nm excitation and emission wavelengths, respectively.

Measurement of mitochondrial transmembrane potential

The mitochondrial transmembrane potential ($\Delta\psi_m$) was measured with 3,3'-dihexyloxacarbocyanine iodide ($\text{DiOC}_6(3)$: excitation $\lambda=484$ nm, emission $\lambda=501$ nm) (Molecular Probes, Inc., Eugene, OR, USA) used at a final concentration of 40 nM in cell suspensions adjusted to 2×10^6 cells/mL. This cyanine dye, which accumulates in the mitochondrial matrix under the influence of the $\Delta\psi_m$, was initially prepared at 1 mM in DMSO (Sigma-Aldrich) and further diluted in distilled water to obtain a 20- μM intermediate solution. After 15 min of incubation at 37°C, $\text{DiOC}_6(3)$ mitochondrial transmembrane potential-related fluorescence was immediately recorded by flow cytometry with a FACScan flow cytometer (Becton Dickinson). The green fluorescence was collected through a 524/44-

nm band pass filter, and the fluorescent signals were measured on a 4-decade logarithmic scale. For each sample, 10,000 cells were acquired and the data were analyzed with LYSYS I software (Becton Dickinson).

Evaluation of apoptotic cells

Nuclear morphology of control and treated cells was studied using fluorescence microscopy after staining with Hoechst 33342 (Sigma-Aldrich). Apoptotic cells were essentially characterised by nuclear condensation of chromatin and/or nuclear fragmentation, as previously described. [12] Three hundred cells were examined for each sample.

Measurement of inflammation

After 24 h of treatment, the supernatants were harvested and stored at -20°C until they were assayed. IL- 1β was determined using ELISA kits (Raybiotech, Inc., Tebu-bio SA, Le Perray-en-Yvelines, France), according to the manufacturer's instructions. The plates were read in an ELISA-reader (Victor IV, Perkin Elmer, Courtaboeuf, France) at 450 nm.

Measurement of intracellular ROS

Intracellular formation of ROS was detected in conjunctival cells with the fluorescent probe 2',7'-dichlorodihydrofluorescein diacetate (DCFH-DA, Molecular Probes, Invitrogen), as we have already described. [10]

Fatty acid composition of cells and mitochondria

Mitochondria were isolated from cell cultures using a commercial kit (Pierce, Perbio, Brebières, France) according to the manufacturer's protocol. Lipids from cells and mitochondria were extracted according to the method developed by Folch et al. [13] Lipids were submitted to fatty acid methylation according to the Morrison & Smith method (1964) and analysed using gas chromatography as previously described. [3]

Statistical analyses

Statistical analyses were performed with the SAS software (SAS Institute, Cary, NC, USA).

ANOVA analysis was used and data were considered statistically different at $p \leq 0.05$.

RESULTS

Fatty acid incorporation in conjunctival cells

BCFAs were not detected in conjunctival Chang cells grown under standard conditions but readily entered the cells when these were incubated with BCFAs (Table 1).

Table 1 about here

We obtained the same results in IOBA cells after addition of isoC16 and isoC20 (23.7% and 8.4% of total fatty acids after 24 h incubation with 100 μ M, respectively) (results not shown). Incubation with C16 significantly increased its endogenous pools in both Chang and IOBA-NHC cells (from +35 to 70% in Chang cells, Table 1). Moreover, BCFAs reached mitochondria, where they accounted for up to one-fourth to one-third of total fatty acids (Table 2). On the contrary, incubation with C16 did not increase its incorporation into mitochondria (Table 2).

Table 2 about here

Cytotoxicity of individual fatty acids

Effect of BCFAs on cell viability

The effect of isoC16, isoC20 and C16 on cell viability was tested using the MTT test, whose results are presented in Table 3. The MTT test reflects mitochondrial dysfunctions by measuring the activity of various mitochondrial dehydrogenase enzymes.

Table 3 about here

There were no differences on mitochondrial SDH activity between the two cell lines ($p = 0.12$) and between the different concentrations of branched-chain fatty acids ($p = 0.16$). However, after 4 h of exposure, there was significantly less SDH activity with isoC20 (45.3–69.2% of the control in Chang cells and 54.1–54.9% in IOBA-NHC cells) and C16 (47.7–67.8% in Chang cells and 58.6–73.1% in IOBA-NHC cells) as compared to the control. After

24 h of exposure, this activity had significantly lowered with isoC20 only (36.0–39.8% of the control in Chang cells and 55.0–56.4% in IOBA-NHC cells).

Fatty acids had no effect on cell growth when compared to control cells, as assessed by protein measurements on cell cultures (data not shown, $p = 0.84$).

BCFAs were not apoptotic

BCFAs did not increase the proportion of propidium iodide-permeable cells and depolarised cells evaluated with flow cytometry ($p = 0.99$ and $p = 0.80$, respectively) or the proportion of apoptotic cells evaluated by Hoechst staining ($p = 0.52$) (Table 4).

Table 4 about here

BCFAs did not induce IL-1 β production

Since BCFAs are increased in blepharitis patients with meibomian gland dysfunction [3] and meibomian gland dysfunction is associated with inflammation at the lid margins [5], we wondered whether the increase in BCFAs influenced conjunctival inflammation. To evaluate the pro-inflammatory effect of BCFA, we measured IL-1 β production after 24 h of treatment. No variation in the production of IL-1 β in Chang and IOBA-NHC cells was observed, even at high doses (100 μ M) of BCFAs (data not shown).

BCFAs did not affect ROS production

ROS production was not modified by BCFAs, as shown in Table 5 ($p = 0.52$).

Table 5 about here

DISCUSSION

The lacrimal film provides the interface between the eye and the outer environment. It is composed of an aqueous layer (7–10 μ m thick) covered by the thin tear film–lipid layer (TFLL, 100 nm thickness) at the external side. [14] The TFLL is secreted by the meibomian

glands and is highly structured. [15] Its main functions are to delay evaporation, maintain a smooth optical surface and provide a barrier against micro-organisms or skin sebum. [5] Significant alterations of the TFLM can lead to a higher rate of tear evaporation, which predisposes to chronic blepharitis and dry eye. [16] Our previous study showed that blepharitis patients with MGD displayed an increase in BCFAs associated with a sharp decrease in saturated fatty acids. [3] Until now, the reason for the increase in BCFAs has remained unknown. Since BCFAs exhibit a lower melting point than the corresponding straight-chain fatty acids, the specific functions of the increase in BCFAs may be enhancing lipid fluidity and providing greater resistance to environmental (evaporation) and biological stresses. [17] The increase in BCFAs may therefore be a beneficial adaptive response of the body counterbalancing the lack of tears by spreading the tear film on the ocular surface. Alternatively, the increase in BCFAs may also have detrimental consequences on conjunctival cells by increasing inflammation and apoptosis and then participating in the vicious circle of blepharitis. Indeed, BCFAs in tears may be toxic for the conjunctiva since conjunctival cells are in permanent contact with lacrimal film and their toxic effects have already been shown in other tissues. [6, 7]

Our results showed no cytotoxic effects of the two BCFAs tested – isoC16 and isoC20 – contrary to treatment with benzalkonium chloride (0.02%), which induced mitochondrial toxicity and apoptosis [18]:

1. BCFAs were not observed to have an effect on morphological aspects of the cells stemming from the concentration tested (50 μ M or 100 μ M), the duration of treatment (4 h or 24 h) or the type of cell line (Chang or IOBA-NHC) (results not shown).
2. BCFAs were not toxic to the plasma membrane, as shown by the nonpermeability of the conjunctival cells to propidium iodide, or to the nucleus, as shown by Hoechst staining.

3. BCFAs did not affect mitochondrial depolarisation. However, only isoC20 induced a significant decrease in mitochondrial SDH activity after 4 h of treatment as well as after 24 h in Chang cells and IOBA-NHC cells. This change did not result from a decrease in the number of cells, as shown by the same protein content. It may be attributable to an alteration in the ability of mitochondria to undergo metabolic changes related to the substantial incorporation of isoC20 in mitochondrial membranes. Indeed, isoC20 accounted for 24.4% of the total fatty acids in mitochondria. This may modify membrane fluidity, leading to changes in SDH activity. IsoC16 was also incorporated into mitochondria in large quantities (36.1% of total fatty acids). However, since it has fewer carbon atoms, it may not increase fluidity as much as isoC20. Moreover, changes in membrane fluidity may regulate certain cellular reactions involving membrane-associated enzymes. For example, an increase in membrane fluidity caused by the incorporation of polyunsaturated fatty acids into membrane increased the activation energy (E_a) of mitochondrial enzymes. [19] Hence, isoC20 may have a direct influence on the SDH active site, contrary to isoC16. We also observed a significant decrease in mitochondrial SDH activity when incubated with C16 for 4 h. The toxicity of saturated fatty acids (70–1000 μ M) has already been observed in other cell types such as endothelial cell monolayers [20] and skeletal muscle cells. [21] The absence of toxicity after 24 h of exposure to C16 means that conjunctival cells were able to detoxify C16, probably by oxidation and without producing toxic metabolites.
4. BCFAs did not induce IL-1 β production. IL-1 β is a cytokine increased in the conjunctival epithelium during dry eye, like other cytokines such as IL-8 and TNF- α . [22, 23] Hence BCFAs were not pro-inflammatory in conjunctival cells.

5. BCFA did not induce ROS production. The production of ROS is a highly controlled process. Excessive ROS production has deleterious effects by initiating lipid peroxidation.

This lack of a toxic effect did not result from these fatty acids not being incorporated. BCFAs were incorporated into the cells' total lipids, between 2 and 7.9% of total fatty acids after 4 h of treatment and 4.5 and 9.6% after 24 h. We studied Chang cells, which are human immortalised cells. [24] Despite their similarity with *in situ* conjunctival epithelium (presence of tight junctions, microvillousities, mucin secretion) and their widespread use in the study of the expression of inflammation-related markers or apoptosis [25], they may behave differently, especially toward toxic molecules, since these cells have HeLa-marker chromosomes. To lessen the impact of the use of the Chang cell line, we verified all the results on IOBA-NHC cells, a nontransfected, spontaneously immortalised epithelial cell line from normal human conjunctiva. These cells retain morphologic and functional conjunctival epithelial characteristics *in vitro*. [26] We found the same results in IOBA-NHC cells as in Chang cells: absence of BCFA and C16 toxicity (viability, apoptosis, inflammation) and incorporation of these fatty acids into cell lipids. This confirms that Chang cells and IOBA-NHC cells are comparable for the *in vitro* toxicological studies, as previously shown. [9]

The increase in BCFAs in the tears of patients with meibomian gland dysfunction had no deleterious effect on conjunctival cells and may instead reflect an adaptive response of the body attempting to increase tear film stability. Indeed, in MGD, meibum is thicker and loses its physical properties required to stabilise the lacrimal film. IsoC20 may have a prominent role, as suggested by our previous study showing a partial, significant restoration of the isoC20 content after minocycline treatment associated with lid hygiene [4], suggesting that the increase in isoC20 may be an adaptive response of the meibomian gland to local changes such as MGD.

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Table 1: Branched-chain fatty acid content in total lipids of Chang cells after 4 h and 24 h of exposure (% of total fatty acids).

	4 h		
	Control <i>n</i> = 3	50 μM <i>n</i> = 3	100 μM <i>n</i> = 3
C16	14.9 \pm 0.91	20.2 \pm 0.42	23.6 \pm 1.21
isoC16	ND	2.0 \pm 0.14	4.9 \pm 1.14
isoC20	ND	5.9 \pm 1.52	7.9 \pm 2.91
	24h		
	Control <i>n</i> = 3	50 μM <i>n</i> = 3	100 μM <i>n</i> = 3
C16	14.6 \pm 0.57	19.9 \pm 1.08	24.5 \pm 0.84
isoC16	ND	4.5 \pm 0.01	9.6 \pm 3.00
isoC20	ND	6.9 \pm 1.35	8.1 \pm 3.64

Data are means \pm SEM and represent three independent experiments.
ND: not detected.

Table 2: Branched-chain fatty acid incorporation in mitochondria of Chang cells after 24 h of exposure (100 μ M) (% of total fatty acids).

	Control	100 μM
C16	10.3 \pm 1.15	11.8 \pm 1.13
isoC16	ND	36.1 \pm 2.26
isoC20	ND	24.4 \pm 2.94

Data are means \pm SEM and represent three independent experiments. ND: not detected.

Table 3: Cell viability in Chang and IOBA-NHC cells after incubation with branched-chain fatty acids, as assessed by measuring SDH activity with the MTT test. Values are expressed as the percentage of control conditions (without incubation with any fatty acids).

4h					
		Chang cells		IOBA-NHC cells	
	Control	50 μ M	100 μ M	50 μ M	100 μ M
C16	100 ^a	67.8 \pm 7.10 ^b	47.7 \pm 4.59 ^c	73.1 \pm 8.50 ^b	58.6 \pm 7.04 ^{bc}
IsoC16	100	101.0 \pm 3.77	99.3 \pm 2.95	104.9 \pm 4.22	101.7 \pm 2.84
IsoC20	100 ^a	45.3 \pm 3.87 ^c	69.2 \pm 2.69 ^b	54.1 \pm 5.66 ^{bc}	54.9 \pm 7.75 ^{bc}
24h					
		Chang cells		IOBA-NHC cells	
	Control	50 μ M	100 μ M	50 μ M	100 μ M
C16	100	89.4 \pm 12.57	80.5 \pm 11.13	83.6 \pm 4.56	68.4 \pm 5.67
IsoC16	100	95.9 \pm 7.16	99.0 \pm 10.38	109.3 \pm 6.09	103.1 \pm 2.05
IsoC20	100 ^a	39.8 \pm 2.45 ^b	36.0 \pm 1.82 ^b	56.4 \pm 2.95 ^b	55.0 \pm 3.64 ^b

Data are means \pm SEM. For the Chang cells, $n = 3-5$ and for the IOBA-NHC cells $n = 3$. On the same row, the means with different superscripts (a, b or c) differ significantly ($p \leq 0.05$).

Table 4: Evaluation of the apoptotic effect of branched-chain fatty acids (100 μ M) incubated for 24 h in Chang and IOBA-NHC cells.

	% of cells permeable to propidium iodide		% of depolarised cells		% of apoptotic cells	
	Chang	IOBA-NHC	Chang	IOBA-NHC	Chang	IOBA-NHC
Control	5.9 \pm 2.34	6.3 \pm 2.85	11.8 \pm 2.85	10.0 \pm 1.00	7.3 \pm 4.07	3.5 \pm 0.50
C16	7.4 \pm 4.12	7.7 \pm 0.33	12.6 \pm 4.84	7.3 \pm 0.66	10.7 \pm 6.78	2.7 \pm 0.60
IsoC16	6.8 \pm 3.93	5.0 \pm 0.00	11.4 \pm 4.42	10.0 \pm 2.00	9.4 \pm 6.75	2.2 \pm 0.73
IsoC20	6.5 \pm 3.44	8.3 \pm 0.33	10.8 \pm 5.25	5.7 \pm 0.33	12.0 \pm 5.51	3.0 \pm 0.29

Percent of propidium iodide-permeable cells was determined by flow cytometry after staining with propidium iodide. Percent of depolarised cells corresponding to cells characterised by a loss of mitochondrial transmembrane potential were determined using flow cytometry after staining with DiOCC₆(3). Percent of apoptotic cells (with fragmented and/or condensed nuclei) was determined after staining with Hoechst 33342. Data are means \pm SEM and represent three independent experiments.

Table 5: Intracellular ROS detection in Chang and IOBA-NHC cells after branched-chain fatty acid treatment (ratio to control).

4h					
		Chang cells		IOBA-NHC cells	
	Control	50 μ M	100 μ M	50 μ M	100 μ M
C16	100	76.9 \pm 10.26	83.2 \pm 5.99	93.1 \pm 10.22	101.5 \pm 10.92
IsoC16	100	100.6 \pm 2.77	86.9 \pm 5.53	90.7 \pm 10.69	92.1 \pm 15.51
IsoC20	100	94.6 \pm 6.24	94.1 \pm 5.73	92.2 \pm 14.75	90.5 \pm 12.68
24h					
		Chang cells		IOBA-NHC cells	
	Control	50 μ M	100 μ M	50 μ M	100 μ M
C16	100 ^{ab}	85.5 \pm 4.85 ^b	94.2 \pm 7.22 ^{ab}	116.3 \pm 10.43 ^a	104.8 \pm 9.47 ^{ab}
IsoC16	100	88.4 \pm 5.44	91.5 \pm 6.88	103.8 \pm 3.61	108.8 \pm 8.59
IsoC20	100	99.4 \pm 12.93	104.6 \pm 9.96	102.0 \pm 8.82	117.2 \pm 8.17

Intracellular formation of ROS was detected in conjunctival cells with the fluorescent probe 2',7'-dichlorodihydrofluorescein diacetate (DCFH-DA). The DCF fluorescence resulting from the oxidation of DCFH-DA was measured with a fluorescence multi-well plate reader. Data are means \pm SEM. For the Chang cells, $n = 3-5$ and for the IOBA-NHC cells $n = 3$. On the same row, the means with different superscripts (a, b or c) differ significantly ($p \leq 0.05$).