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On the function of pentadecanoic acid and docosahexaenoic acid during culturing of the thraustochytrid, *Aurantiochytrium* sp. NB6-3

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Abstract

During culturing of *Aurantiochytrium* sp NB6-3, the maximum triglyceride (TG) content was obtained at the end of logarithmic phase, after which it rapidly decreased until the mid-stationary phase. Of these TGs, pentadecanoic acid (C15), palmitic acid (C16) and docosahexanoic acid (DHA) were major fatty acids. Interestingly, the unusual fatty acid-C15 reached a maximum content of 22mg/g lyophilized cells at the end of the logarithmic phase, after which it decreased to 6 mg/g cells at the end of the culture. The variation in the C16 content was similar to that in C15 content. On the other hand, the variation in DHA content followed the trend of C15 until 60 h, then reached a minimum value at 72 h, after which it increased again to finally reach to up to 30 mg/g cells at the end of the culture. To better understand the function of C15 and DHA in TG, *Aurantiochytrium* sp NB6-3 was cultured in growth media containing fatty acid mixture of DHA and C15, DHA and myristic acid (C14), C15 and oleic acids (C18=1), or C14 and C18=1, respectively. In C15 and/or DHA-containing media, the cell growth was stimulated. The cell growth was most stimulated in growth media containing both DHA and C15. When BalB/3T3 mouse fibroblasts were cultured with mixtures of C15 and DHA or mixtures of C18=1 and C15, similar results were obtained. These results showed that DHA and C15 synergistically stimulated the cell growth of *Aurantiochytrium* sp. and mouse fibroblasts.

Keywords: pentadecanoic acid; docosahexaenoic acid; synergistic effect; cell-growth effect; *Aurantiochytrium* sp.; mouse fibroblasts

Introduction

Aurantiochytrium sp., a marine thraustochytrid with a high growth rate, has two fatty acid synthesis path ways [1]. One pathway contains the fatty acid synthase and is used for the synthesis of saturated fatty acids, whereas the other pathway contains the polyketide synthase without desaturases and is used for the synthesis of polyunsaturated fatty acid. Pentadecanoic acid (C15) and palmitic acid (C16), and docosahexaenoic acid (DHA) are major fatty acids of thraustochytrids including *Aurantiochytrium* [2]. The saturated fatty acids C15 and C16 are synthesized by the pathway containing fatty acid synthase, whereas DHA is synthesized by the pathway containing polyketide synthase, respectively.

In living cells, the fatty acid moiety of triglycerides is used for energy production through the formation of acetyl-CoA by β-oxidation. From even-numbered fatty acids (EFAs), only acetyl-CoA is formed by β-oxidation, whereas from odd-numbered fatty acids (OFAs) such as C15, acetyl-CoA and propionyl-CoA are formed. Although they have some biological function, OFAs are unusual fatty acids. The hair glowing effect of C15-monoglyceride, which was due to propionyl-CoA derived from C15 by β-oxidation, has been demonstrated in hair follicles of rabbits [3]. In addition, OFAs have been used for anaplerotic therapy for Alzheimer's disease [4, 5], cardiac disorder [6] and cancer [7]. Interestingly, the metabolic conversion from propionyl-CoA to methyl malonyl-CoA closely relates with anaplerotic reactions. Previous studies analyzed anaplerosis from propionate in rat heart perfused with 0-2 mM [^{13}C-3] propionate and the physiological

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concentrations of glucose, lactate and pyruvate, and concluded that [^{13}C-3] propionate acts as a pure anaplerotic substance [8, 9]. Propionyl-CoA is converted to methyl malonyl-CoA, which is subsequently converted to succinyl-CoA by the enzyme activity of methyl malonyl-CoA mutase in a vitamin B_{12}-dependent manner. When succinyl-CoA, a member of the TCA cycle, is converted to succinic acid, GTP/ATP is produced [10]. GTP is a well-known signal transduction substance, which, through the action of G-proteins, regulates multiple physiological functions [11]. On the other hand, methyl malonyl-CoA, the key metabolite derived from propionyl-CoA, is an intermediate of branched-chain amino acid catabolism [12, 13]. From the OFA metabolism closely related with anaplerosis (Figure 1), the function of EFA is considerably different from that of OFA.

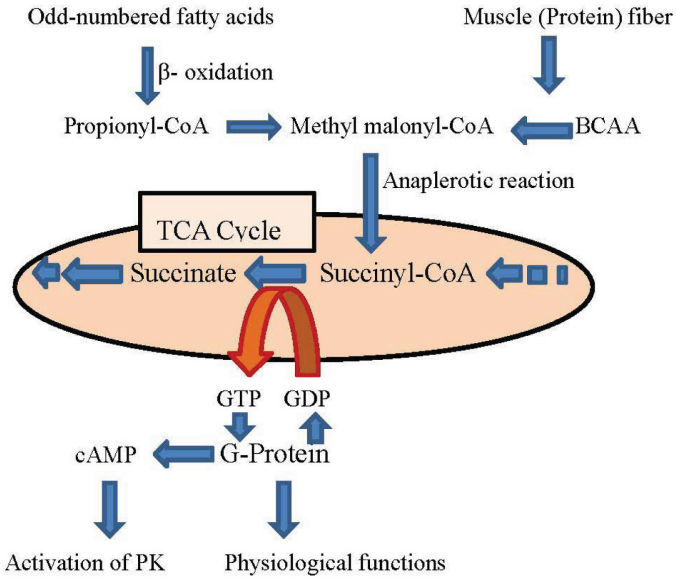

Figure 1 Metabolism of odd-numbered carbon fatty acids. Anaplerotic reaction is the pathway from methyl malonyl-CoA to succinyl-CoA by methyl malonyl-CoA mutase with vitamin B_{12} as the co-factor.

Abbreviations: BCAA = Branched carbon chain amino acids; PK = Protein kinase

The ω -3 fatty acid DHA is an essential fatty acid for human and animal health of which, the multiple functions including anti-inflammation, blood pressure-reducing effect [14], decrease in risk of food allergy [15] and improvement of cardiovascular function [16, 17] have been examined. These functions are most probably due to an effect on the molecular fluidity of biomembranes and an anti-oxidant effect, which are caused by the presence of six double bonds in the DHA molecule [18].

Although DHA is a major fatty acid of *Aurantiochytrium* that is distributed in phospholipids (PLs) and triglyceride (TG) molecules [19], the functions of OFA and DHA in *Aurantiochytrium* are still unknown.

Previously, remarkable variations in the content of major fatty acids as C15, C16 and DHA in TG were found during culturing of *Aurantiochytrium* sp in growth medium. To investigate the effect of C15 and DHA on cell growth, *Aurantiochytrium* sp. NB6-3 cells and BalB/3T3 mouse fibroblasts were cultured in C15- and DHA- containing growth medium, respectively. The present study suggests a synergistic effect of C15 and DHA on cell growth.

Materials and methods

Materials

Aurantiochytrium sp. NB6-3 was obtained from OP Bio factory Inc. (Uruma, Okinawa, Japan). Mouse fibroblast, BALB/3T3 clone A31 (Cell No.RCB0005), was purchased from Riken Bioresource Center (Tsukuba, Japan). Yeast extract and tryptone were purchased from Becton, Dickinson and Company (Sparks, MD USA). D-Glucose, sea salt (Red Sea Salt, Red Sea Salt USA, Houston, TX, USA), myristic acid (C14) and silica gel-60 were obtained from Wako Pure Chemicals (Osaka Japan). C15 and DHA were obtained from Tokyo Chemical Industry Co. Ltd (Tokyo, Japan). Methyl esters of saturated and unsaturated fatty acids were obtained from GL-Science, Tokyo, Japan. Solvents and reagents were of analytical grade.

Culture conditions

Aurantiochytrium sp., strain NB6-3, was grown in 500 mL Sakaguchi culture flasks containing 250 mL of GTY medium (1% tryptone, 2% glucose, 0.5% yeast extract and 1.0% sea salt) as described earlier [20]. The cells were grown isothermally at 25°C at 100 reciprocal strokes/min and were harvested at each 12 h interval by centrifugation at 2,500 g for 15min after which they were washed twice with a 1.5% sea salt solution. The packed cells were lyophilized and the amount of cell growth was obtained by weighing the lyophilized cells.

Lipid extraction

Lipids were extracted from the lyophilized cells using a mixture of chloroform/methanol (2:1, v/v). Individual lipid classes were separated by chromatography on a silica gel 60 column using chloroform. Neutral lipids were eluted by 4 bed volumes of chloroform. After the elution, polar lipids were eluted with 4 bed volumes of chloroform/methanol (1:4, v/v). TGs were fractionated by chromatography using silica gel-60. The column was prepared with n-hexane. The neutral lipids fraction was dissolved with n-hexane and applied to the column. Hydrocarbons, such as squalene, non-polar carotenoids, and sterol esters were eluted with 2 bed volumes of n-hexane/chloroform (1:1, v/v). After the elution, TG, polar carotenoids and free fatty acid were eluted with 3 bed volumes of chloroform. To purify TG, the TG fraction was applied to preparative TLC on silica gel-60 plates using n-hexane/diethyl ether/acetic acid (82/18/1, v/v). TG migrated around Rf 0.6 - 0.8 by using of the developing solvent. The spot on TLC was visualized by spraying with water after which it was scraped off from the plates. TGs were extracted with chloroform/methanol (1:1, v/v). The TG content in the fraction was above 96% by weight.

PLs were fractionated by preparative TLC on silica gel-60 plates using chloroform/ methanol/ acetic acid/ water (25/15/4/2, v/v). PLs migrated around Rf 0.2-0.8 by using of the developing solvent. The spots on TLC were detected by spraying with Zinzadze reagent [21] and visualized by spraying with water, PLs spots were scraped off and extracted with chloroform/methanol (1:5, v/v). PLs content in the polar lipid fraction was above 92% by weight.

Fractionation of TG consisting of only saturated fatty acids

After removal of the extraction solvent, the remaining TG was re-dissolved with 5 volumes of n-hexane after which the solution was stored at 0 – 4°C for overnight. After storage, white precipitates formed which were washed with a small amount of chilled n-hexane. The solvent fraction was condensed at 35°C with a stream of nitrogen and the precipitation procedure was repeated twice. From the solvent fraction, the polyunsaturated fatty acid-containing TG was obtained. TG spots on TLC plates were made visible by spraying with 1% iodine ethanol solution, water or charred after spraying 20% H_2SO_4 (v/v).

Fatty acid analysis

Fatty acids of TG and PLs were converted to methyl esters by reacting them with 14% boron trifluoride in methanol at 90°C for 15 min. The fatty acid methyl esters were extracted with n-hexane and then analyzed by gas-liquid chromatography with flame ionization detector (Shimadzu Corp. Kyoto, Japan) using an open tubular glass column (ϕ 3 mm×3 m) with Unisole 3000 coated on Uniport C 80/100 (GL-Science, Tokyo, Japan).

Cell growth test for Aurantiochytrium sp. NB6-3

DHA (50 mg) and C15 (25 mg) were dissolved in1.0 mL 50% dimethyl sulfoxide (DMSO) aqueous solution. Nitrogen gas bubbled-suspension was homogenized by ultrasonication. Oleic acid (C18=1) (50 mg) and C14 (25 mg) were used for the preparation of emulsions of fatty acids mixture instead of DHA and C15, respectively. The mixtures were diluted ten times with GTY growth medium. Pre-cultured cells (10^5cells in 100 μL GTY growth medium) were supplied to each well of a 96 well microplate after which10 μL of each of the diluted fatty acid mixtures was added to each well. As a control, 50% DMSO aqueous solution was used as described above. The final concentration of DMSO in the growth medium was 0.5%. The plates were incubated at 25°C for 24 h. After incubation, cell growth was measured by turbidity at 650 nm using a microplate reader.

Cell growth test for BalB/3T3mouse fibroblasts

Fatty acid solutions were prepared as described above and diluted to 10 or 100 times with 50% DMSO aqueous solution. The original fatty acid solution and the dilute 10 times with Dulbeco' Modified Eagle's medium, and added to the medium. In 100 μL of the medium, the final C15 concentration was, 0.25 μg, 2.5 μg and 25 μg whereas the final DHA or C18=1 concentration was, 0.5 μg, 5.0 μg and 50 μg. Cells were inoculated at a density of 10^4 cells/well and incubated for 24 h at 37°C in 5% CO_2. After incubation, 10 μL of the cell counting reagent CCK-8 (Dojindo Molecular Technologies Inc. Kumamoto, Japan) was added to each well and the cells were incubated for 4 h at 37°C in 5% CO_2. After incubation, the cell concentration was determined using a microplate reader at 450 nm.

Results and discussion

To investigate the metabolic inter-relationship between OFAs, EFAs and DHA in TG during cell culture, the content and fatty acid composition of TGs and PLs were determined. Due to its short lag time and industrial usage, *Aurantiochytrium* sp.NB6-3 was selected. Usually, the lag time of *Aurantiochytrium* in growth medium is 1-2 days. The growth curve of the *Aurantiochytrium* cells is shown in Figure 2 (culture conditions are 250ml GTY medium in 500 mL Sakaguchi's flask; Temperature: 25°C; Shaking: reciprocal shaker, 100 strokes/ min). After a short lag phase, the cells started to grow and reached the end of the logarithmic phase at 36 h. The maximum biomass was observed at 60 h with a biomass amount 4.4g/L. After 60 h, the biomass gradually decreased until 156 h. However, in this culture period, the death phase was not observed.

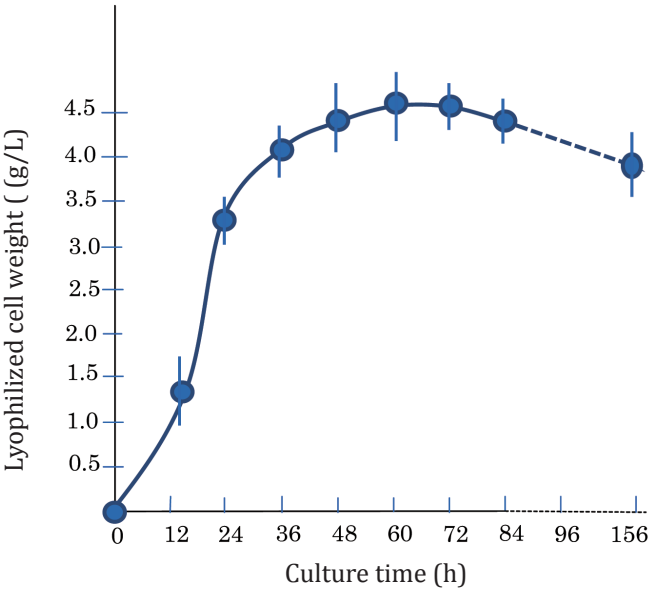

Figure 2 Growth curve of Aurantiochytrium sp. NB6-3 cells. Values are averages of three or more independent experiments from pooled samples. Each standard deviation (SD) is expressed as bar.

Changes in the content of TG and PLs in cells during culture

For the pre-culture, cells were cultured for 24 h in the same medium and the same conditions as those used for the experimental cultivation. For the experimental cultivation, 2.5 mL of pre-cultured cells were inoculated in 250 mL medium. As shown in Figure 3, the TG content (% in lyophilized cells) rapidly increased after the inoculation and stopped to increase at 48 h. Since the TG content reached a maximum just before the maximum cell number was reached, the increasing TG trend correlated

well with the logarithmic phase of the cell culture. At 48 h, the TG content was 10 % of the cells mass. After the maximum was reached, the content rapidly declined to 4 % of cells mass at 72 h and sustained at this low level until the end of the cultivation. These results suggest that changes in the TG content relate with cell growth. On the other hand, PLs content (44.5±4.67 mg/g lyophilized cells) was constant at 4.5±0.5% of the cells throughout the cultivation. However, when the TG content reached the maximum at 48 h, the PLs content decreased to a minimum. It is suggested that this minimum PLs content might be due to the maximum content of TG.

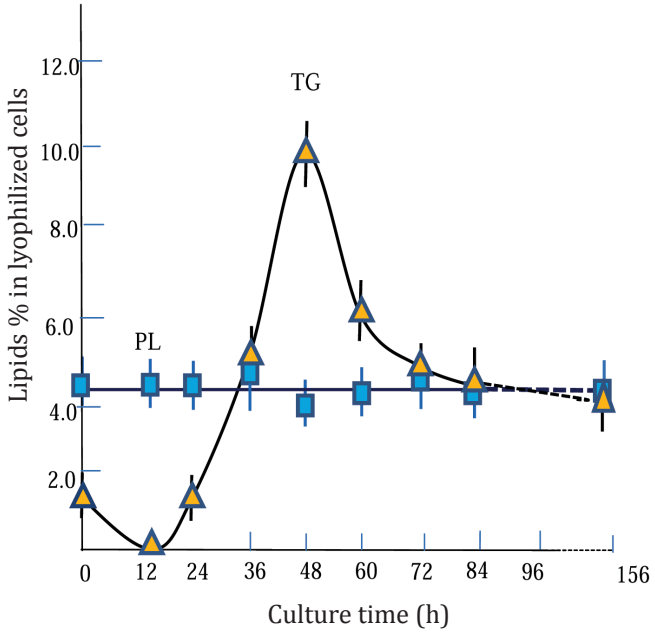

Figure 3 Changes in triglyceride and phospholipid contents in cells during culture. Values are averages of three or more independent experiments from pooled samples. Each SD is expressed as bar. Abbreviations: TG = triglyceride; PL = phospholipid.

The fatty acid compositions of TG at 12 h, 24 h, 48 h, 72 h, and 156 h are shown in Table 1. During the cultivation, extensive fluctuations in C15 and DHA contents were observed in TG. In fact, the C15 content was 3.3% at 12 h, which increased to 24.5% at 72 h and declined to 6.0% at 156 h. The initial value of DHA was 27.5%, which decreased to 16.2% at 48 h after which the value increased again to reach to 70.5% at 156 h.

When expressed as fatty acid content per mass of lyophilized cells, the trends of the fatty acids contents during the culture are obvious (Figure 4). With increasing TG levels, the contents of C15, C16, and DHA in TG increased accordingly in the lyophilized cells. After 48 h, C15 and C16 decreased in parallel with decreasing TG levels. Whereas C15 and C16 are synthesized by the pathway containing fatty acid synthases, DHA is synthesized by the pathway containing polyketide synthases [1]. During the logarithmic phase, both of these pathways were active. After the logarithmic phase, when the TG content of the cells started to decrease, only the pathway containing polyketide synthase was still active, whereas the pathway containing fatty acid synthase was almost inactive.

Table 1 Changes in fatty acid composition of triglyceride during culture.

Fatty acid	*Culture time (h)*				
	12	*24*	*48*	*72*	*156*
C_{12}	1.8	0.8	0.8	0.2	3.4
C_{13}	tr[a]	0.8	1.9	1.0	3.4
C_{14}	18.9	16.6	21.4	6.0	2.4
C_{15}	3.3	9.8	21.0	24.5	6.0
C_{16}	43.5	41.0	37.5	27.4	12.2
C_{17}	tr	0.6	0.2	8.7	2.1
C_{18}	0.8	0.8	0.3	0.1	tr
$C_{22=5}$	4.2	1.0	0.7	0.8	tr
$C_{22=6}$	27.5	28.6	16.2	31.3	70.5

Note: Values are expressed as weight % of total peak area and are averages of three or more independent experiments from pooled samples. Details of isolation and GC analysis of the fatty acids are given under Materials and methods. [a]tr represents values < 0.1%.

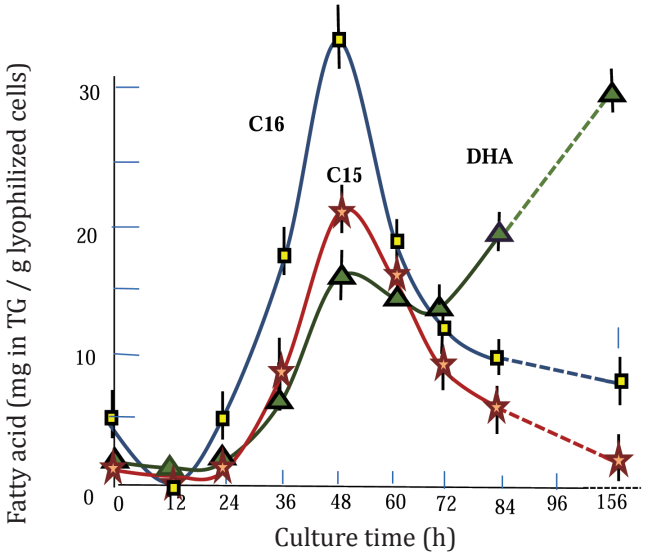

Figure 4 Changes in major fatty acids contents in triglyceride in cells during culture. Values are averages of three or more independent experiments from pooled samples. Each SD is expressed as bar. *Abbreviations:* TG = triglyceride; C15 = pentadecanoic acid; C16 = palmitic acid; DHA = docosahexaenoic acid.

Changes in fatty acid composition of PLs are shown in Table 2. Although the compositions fluctuated during the cultivation no huge changes were observed. Docosapentaenoic acid (C22=5) was distributed in PLs and gradually increased from 10.0 % to 29.4% during the cultivation, while the combined content of C22=5 and DHA was almost constant. Considering that DHA and C22=5 have the same physical characteristics and that PLs are major components of lipid bilayer of biomembranes, these results suggest that the cell membrane fluidity remained almost constant throughout the culturing period.

Fractionation of TG that contain only saturated fatty acids

When the TG isolated at 48 h was dissolved with n-hexane and stored at 4^{0}C for overnight, a white precipitate was

Table 2 Changes in fatty acid composition of phospholipid during culture.

Fatty acid	*Culture time (h)*				
	12	*24*	*48*	*72*	*156*
C_{12}	0.1	0.1	0.2	0.1	tr[a]
C_{13}	tr	0.1	0.1	tr	tr
C_{14}	5.4	2.2	2.8	0.4	0.7
C_{15}	1.6	3.5	7.7	9.6	5.2
C_{16}	45.0	33.3	27.1	26.0	27.0
C_{17}	tr	0.3	0.9	5.5	6.4
C_{18}	0.6	0.9	0.3	tr	0.2
$C_{22=5}$	10.0	11.1	18.5	21.0	29.4
$C_{22=6}$	37.3	48.5	42.4	37.4	31.1
$C_{22=5}$+ $C_{22=6}$	47.3	59.6	60.9	58.4	60.5

Note: Values are expressed as weight % of total peak area and are averages of two or more independent experiments from pooled samples. Details of isolation and GC analysis of the fatty acids are given under Naterials and methods. [a]tr represents values < 0.1%.

observed which was collected by centrifugation. After washing the precipitates using cold n-hexane, the purified precipitates and the soluble fraction were dissolved with chloroform and applied on TLC plates using chloroform/ methanol/ acetic acid/ water (25/15/4/2, v/v) as the developing solvent. After development, the plates were sprayed with 20% H2SO4 followed by heating, or exposed to iodine vapor. However, on the TLC, the spot of the precipitate fraction on the TLC was negative against both reagents, whereas the soluble fraction was positive against both. The fatty acid compositions of both fractions are shown in Table 3. The major fatty acids of the white precipitates fraction (solid-TG) were the saturated fatty acids C16, C14 and C15. This solid-TG fraction could be formed only in TG isolated from cells grown up to 72 h after the start of the culture. The major fatty acids of TG in the soluble fraction (liquid-TG) were DHA, C16, C15 and C14. In this liquid-TG fraction, the DHA content was 2.7 times higher than in the original TG. These findings indicate that at least two types of TG exist in this organism. One type consists of only saturated fatty acids (solid-TG), whereas another type is TG containing DHA (liquid-TG), solid-TG consisted of only saturated fatty acids including OFAs and EFAs, which are synthesized by the pathway containing fatty acid synthase, whereas liquid-TG consisted of polyunsaturated fatty acids synthesized by the pathway containing polyketide synthase [1], Based on the DHA contents of non-treated TG and liquid-TG, the ratio of solid-TG and liquid-TG was 63/37 when TG production was at its maximum. However, as the fractionation was not perfect, we assumed that a small amount of solid-TG was still remaining in the liquid-TG fraction. After reaching the maximum ratio of solid-TG and liquid-TG, the ratio shifted to 42/58 at 72 h. At the end of the culture, the DHA consisted of 70.5% of the total fatty acids in the non-treated TG, whereas solid-TG could not be formed. It is suggested that solid-TG might be replaced by liquid-TG. From the results, we assumed that solid-TG and liquid-TG produced in logarithmic phase play an important part in the cell growth. Especially, OFAs in solid -TG produced in logarithmic phase ought to be converted to acetyl-CoA and propionyl-CoA by β-oxidation. Propionyl-CoA acts as an anaplerotic substance [10] (Figure 2). These reactions must be related to the cell growth. Also, DHA in liquid -TG produced in logarithmic phase may be related to the cell growth. After the logarithmic phase, DHA content in TG increased again. This increase must be related with oxidative stress of cells.

Table 3 Fatty acid compositions of solid and liquid fractions of triglyceride (TG) isolated from cells cultured for 48 h.

Fatty acid	*Fatty acid composition (%)*		
	Non-treated TG	*Solid-TG*	*Liquid-TG*
C_{12}	0.8	0.3	1.0
C_{13}	1.9	1.0	1.6
C_{14}	21.4	23.3	14.3
C_{15}	21.0	22.9	13.9
C_{16}	37.5	52.4	23.2
C_{17}	0.2	0.2	tr[a]
C_{18}	0.3	tr	0.4
$C_{22=5}$	0.7*	tr	1.6
$C_{22=6}$	16.2	tr	43.8
*Ratio (%)	100	63	37

Note: Values are expressed as weight % of total peak area and are typical of data from several replicate experiments.

Abbreviations: $C_{22=6}$ = DHA; $C_{22=5}$ = DPA; [a]tr represents values < 0.1%; TG = triglyceride; *Ratio was calculated based on the $C_{22=6}$ contents of non-treated TG and liquid-TG.

Effects of the fatty acid mixture on cell growth of Aurantiochytrium sp. NB6-3 and BalB/3T3 mouse fibroblast

To clarify the function of the increasing C15 and DHA levels, cells were grown in growth medium containing different fatty acid mixtures. The fatty acid C16 is a major fatty acid in TG of the cells, and is metabolized by β-oxidation to form 8 molecules of acetyl-CoA .The fatty acid-C15 is converted to 6 molecules of acetyl-CoA and one molecule of propionyl-CoA. Instead of C16, C14 was used in the current experiments to make up for the number of acetyl-CoA plus propinyl-CoA molecules produced from original fatty acids.

As shown in Table 4, the media containing the mixture of DHA and C15 and the mixtuew of DHA and C14 had a remarkable effect on cell growth. However, the mixture of DHA and C15 exerted a higher effect on cell growth than did the mixture of DHA and C14. Although the mixture of

C18=1 and C15 also stimulated cell growth, the magnitude was lower than that of the mixture of DHA and C15.

Table 4 Effect of fatty acid mixture on cell growth of Aurantiochytrium sp.NB6-3.

Sample (fatty acids /100μL medium)	*Cell growth ratio* (n=6)*
Control (0.5% DMSO)	100.0±14.6
DP-1 (DHA, 50 μg +C15, 25 μg)	†**184.6±10.0
DM-1 (DHA, 50 μg +C14, 25 μg)	†**138.3±10.6
OP-1 (C18=1, 50 μg +C15, 25 μg)	**124.8±7.1
OM-1 (C18=1, 50 μg +C14, 25 μg)	118.2±10.2

Note: The average of the control values was expressed as 100. *Cell growth ratio = [(turbidity after culture for 24 h) −(turbidity before culture)] /[(turbidity of control after culture for 24 h) −(turbidity of control before culture)] ×100. Turbidity was measured at 650 nm. ** p <0.01; †p <0.01 (between DP-1 and DM-1).

To confirm the universality of the cell growth stimulation by the mixture of C15 and $C_{22=6}$, mammalian fibroblasts were tested. When BalB/3T3 mouse fibroblasts were cultured with mixtures of DHA and C15 or mixtures of $C_{18=1}$ and C15, similar results were obtained (Table 5). After dilution of the fatty acid solution in growth medium, the final C15 concentration was 0.25 μg, 2.5 μg, and 25 μg whereas the final DHA or $C_{18=1}$ concentration was 0.5 μg, 5.0 μg , and 50 μg in 100 μL of medium. Of the mixture of DHA and C15, a significant effect on cell growth was observed in the maximum and middle doses. Of the mixture of $C_{18=1}$ and C15, a significant effect on cell growth was observed in the maximum dose. These results were similar to those observed in the cell growth experiments of *Aurantiochytrium* sp. NB6-3.These indicated that both DHA and C15 have a stimulating effect on cell growth of *Aurantiochytrium* sp. and mouse fibroblasts. When both fatty acids were added to the medium, the cell growth was stimulated synergistically. Upon β-oxidation of C15, propionyl-CoA and acetyl-CoA are formed. Propionyl-CoA, a known anaplerotic substance [8, 9], is converted to methyl malonyl-CoA which is subsequently converted to succinyl-CoA by the enzyme activity of methyl malonyl-CoA mutase in a vitamin B12-dependent manner. Succinyl-CoA is converted to succinate in the TCA cycle resulting in GTP production [10]. As such, through the anaplerotic reaction, it is assumed that the stimulatory effect of DHA and C15 on fibroblast growth could be related to increased bioenergetics of the cells [3, 22]. In parallel, the physiological effects of DHA may be dependent on increasing the cell membrane fluidity [14], and anti-oxidation effects [18].

Conclusion

Both DHA and C15 stimulated cell growth of *Aurantiochytrium* sp. and mouse fibroblasts. The cell growth showed a synergistic effect when the mixture of DHA and C15 was used. The findings suggested that the cell growth stimulation of fibroblasts may be utilized for dermis reclamation in the near future.

Table 5 Effect of fatty acid mixture on cell growth of BalB/3T3 mouse fibroblast.

Sample (fatty acids /100μL medium)	*Cell growth ratio (n=7)*
Control (0.5% DMSO)	100.0±6.6
DP-1 (DHA, 50 μg +C_{15}, 25 μg)	*126.3±7.3
DP-2 (DHA, 5.0 μg +C_{15}, 2.5 μg)	*109.0±3.3
DP-3 (DHA, 0.50 μg +C_{15}, 0.25 μg)	101.0±5.1
OP-1 ($C_{18=1}$, 50 μg +C_{15}, 25 μg)	*111.4±7.2
OP-2 ($C_{18=1}$, 5.0 μg +C_{15}, 2.5 μg)	105.2±5.9
OP-3 ($C_{18=1}$, 0.50 μg +C_{15},0.25 μg)	98.6±10.9

Note: The average of the control values was expressed as 100. Cell growth ratio was calculated similar as that described for Table. *p <0.01.

Acknowledgements

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Conflict of interest

Authors declare that no conflict of interest.

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