

## Combination therapy with butyrate and docosahexaenoic acid for keloid fibrogenesis: an in vitro study\*

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**Abstract:** BACKGROUND: A single, effective therapeutic regimen for keloids has not been established yet, and the development of novel therapeutic approaches is expected. Butyrate, a short-chain fatty acid, and docosahexaenoic acid (DHA), a  $\omega$ -3 polyunsaturated fatty acid, play multiple anti-inflammatory and anticancer roles via their respective mechanisms of action.

OBJECTIVE: In this study, we evaluated the antifibrogenic effects of their single and combined use on keloid fibroblasts.

METHODS: Keloid fibroblasts were treated with butyrate (0-16 mM) and/or DHA (0-100  $\mu$ M) for 48 or 96 h.

RESULTS: Butyrate inhibited cell proliferation, and  $\alpha$ -smooth muscle actin ( $\alpha$ -SMA) and type III collagen expressions, with inhibition of the transforming growth factor (TGF)- $\beta$ 1 and TGF- $\beta$  type I receptor expressions and increased prostaglandin E<sub>2</sub> with upregulation of cyclooxygenase-1 expression with induction of histone acetylation. DHA inhibited  $\alpha$ -SMA, type III collagen, and TGF- $\beta$  type I receptor expressions. Then, the butyrate/DHA combination augmented the antifibrogenic effects, resulting in additional inhibition of  $\alpha$ -SMA, type I and III collagen expressions, with strong disruption of stress fiber and apoptosis induction. Moreover, the butyrate/DHA combination inhibited the cyclooxygenase-2 expression, suggesting stronger anti-inflammatory effect than each monotherapy.

STUDY LIMITATIONS: Activation in keloid tissue is affected not only by fibroblasts but also by epithelial cells and immune cells. Evaluation of the effects by butyrate and DHA in these cells or in an in vivo study is required.

CONCLUSION: This study demonstrated that butyrate and docosahexaenoic acid have antifibrogenic effects on keloid fibroblasts and that these may exert therapeutic effects for keloid.

**Keywords:** Butyrates; Docosahexaenoic acids; Fibroblasts; Fibrosis; Keloid; Prostaglandins E

### INTRODUCTION

Keloid and hypertrophic scars are dermal fibroproliferative disorders unique to humans, causing cosmetic deformities and psychological stress, which consequently impair the patient's quality of life.<sup>1</sup> Current treatment modalities such as surgery, radiation, and immunomodulation have been demonstrated to have therapeutic effects, but no modality has completely eliminated the risk of recurrence.<sup>2,3</sup>

Keloid formation has been linked to aberrant fibroblast activity resulting in increased expression of many potent cytokines and growth factors, especially transforming growth factor (TGF)- $\beta$ 1.<sup>4</sup> The binding of TGF- $\beta$ 1 to TGF- $\beta$  receptors, including TGF- $\beta$  type I receptor (TGF- $\beta$ RI) and TGF- $\beta$  type II receptor (TGF- $\beta$ RII), leads to activation of the TGF- $\beta$ RI by TGF- $\beta$ RII-mediated phosphorylation.<sup>5</sup> As a result of the response to TGF- $\beta$ 1, cellular recruitment of the fibroblasts is enhanced, resulting in fibroblast differentiation into myofibroblasts; the differentiation causes intense cell proliferation, apoptosis resistance, and synthesis of extracellular matrix proteins, especially type I collagen (collagen I) and type III collagen (collagen III).<sup>6</sup> In fibroproliferative tissues, fibroblast differentiation into

myofibroblasts is commonly identified by  $\alpha$ -smooth muscle actin ( $\alpha$ -SMA) expression, which is altered by stress fiber formation in the downstream signaling of TGF- $\beta$ 1.<sup>7</sup> Functionally, these cells can generate contractile force and promote fibrosis. Therefore, the suppression of these parameters may be useful in therapeutic approaches for keloids. In addition, keloid fibroblasts (KFBs) secrete less prostaglandin E<sub>2</sub> (PGE<sub>2</sub>) than normal dermal fibroblasts (NFBs),<sup>8</sup> and PGE<sub>2</sub> has been reported to possess antifibrogenic activities such as inhibition of fibroblast proliferation, migration, and collagen expression in KFBs.<sup>8,9</sup>

Short-chain fatty acids (FAs) are the endproducts of anaerobic bacterial fermentation of dietary fibers in the colon. These FAs, predominantly butyrate and propionate, have a histone deacetylase (HDAC) inhibitor activity and play multiple roles such as apoptosis induction, proliferation regulation, and differentiation in the colonic epithelium.<sup>10,11</sup> As for the effects of short-chain FAs on PGE<sub>2</sub> secretion, we have reported that butyrate and trichostatin A, a typical HDAC inhibitor, indicates more PGE<sub>2</sub> secretion and stronger atten-

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uation of nuclear factor- $\kappa$ B activation than propionate in lipopolysaccharide-activated human peripheral blood mononuclear cells.<sup>12</sup> Butyrate or other HDAC inhibitors have been reported to have antifibrogenic effects on several mesenchymal cells, including human lung fibroblasts, showing inhibition of cell proliferation, collagen production, and  $\alpha$ -SMA expression through histone acetylation.<sup>13-15</sup> In our study using NFBs, butyrate exerted stronger antifibrogenic effects than propionate.<sup>16</sup> Moreover, butyrate was reported to be a more potent HDAC inhibitor than propionate.<sup>10</sup> However, these effects of butyrate on KFBs have not been investigated.

Docosahexaenoic acid (DHA) and eicosapentaenoic acid are major FAs in  $\omega$ -3 polyunsaturated FAs of marine organisms, including fish oil. These FAs have anti-inflammatory and anticancer effects.<sup>17,18</sup> Antifibrogenic effects of DHA have been reported in human peritoneal fibroblasts, including inhibition of TGF- $\beta$ 1, vascular endothelial growth factor, and collagen I expressions.<sup>19</sup> In our study using NFBs, DHA exerted stronger antifibrogenic effects than eicosapentaenoic acid, indicating inhibition of  $\alpha$ -SMA, TGF- $\beta$ 1, and collagen III expressions, in concurrence with a report on human prostate carcinoma cell line.<sup>16,20</sup> DHA is a ligand of peroxisome proliferator-activated receptor  $\gamma$  (PPAR $\gamma$ ), and lipid mediators derived from DHA are potent agonists of PPAR $\gamma$ .<sup>21</sup> Many studies reported that PPAR $\gamma$  expression or activation inhibited collagen I,  $\alpha$ -SMA, and TGF- $\beta$ RI expressions in mouse skin and embryonic and human dermal fibroblasts.<sup>21,22</sup> Therefore, PPAR $\gamma$  expression regulation by DHA might be a possible underlying mechanism of antifibrogenic effects. However, these effects of DHA on KFBs have not been investigated.

Although we have reported that butyrate and DHA showed inhibition of profibrotic factors in NFBs, the effect of these FAs in KFBs remain unclear. In the present study, we investigated the antifibrogenic effects of single and combined treatment with butyrate and DHA on KFBs by measuring profibrotic factors, cell proliferation, apoptosis, and stress fiber formation, and the underlying mechanism of the antifibrogenic effects on each FA by measuring PGE<sub>2</sub> secretion, histone acetylation, and PPAR $\gamma$  expression level.

## METHODS

### *Primary keloid fibroblast cultures*

KFBs were obtained from chest and earlobe keloid tissues of two Japanese patients. The protocol for tissue collection was approved by the ethics review board at Kobe University Graduate School. Five or 6 pieces of the minced and deepithelialized samples were placed on a 100-mm tissue culture dish (Iwaki, Tokyo, Japan) and immersed in 2 mL Dulbecco's modified Eagle medium (DMEM; Wako Pure Chemical Industries, Osaka, Japan) supplemented with 10% fetal bovine serum (FBS; Nichirei, Tokyo, Japan), penicillin (50 U/mL), and streptomycin (50  $\mu$ g/mL; MP Biomedicals, Illkirch, France). On the following day, 15 mL of the medium was added to each dish. The culture medium was changed every 2 or 3 days until approximately 80% confluence was reached. The cells were passaged by incubation at 37°C with 0.05% trypsin and 0.02% EDTA, and plated in culture dishes.

### *Cell culture*

KFBs were grown at a 37°C CO<sub>2</sub> incubator, using DMEM with 10% FBS. Only cells from passage 3 to 6 were used in this ex-

periment. Trypan blue staining was performed to distinguish live cells from dead cells and absolute cell counts. For experimentation, KFBs were seeded into 6-well flat bottom plates (Iwaki) at a concentration of  $2.8 \times 10^5$  cells/well and 96-well flat bottom plates (Iwaki) at a concentration of  $8.0 \times 10^3$  cells/well.

### *FA treatment*

Sodium butyrate (Sigma, St. Louis, MO) and sodium DHA (Sigma) were used. To evaluate the effects of the monotherapy, butyrate or DHA was applied at 0, 4, and 16 mM or 0 and 100  $\mu$ M, respectively, after our experiments on NFBs.<sup>16</sup> To evaluate the combination treatment, butyrate was applied at 4 and 16 mM with DHA at 100  $\mu$ M.

### *BrdU assays*

KFBs were seeded into 96-well flat bottom plates and cultured in culture medium with 10% FBS. After 24 h, FAs were added and the cells were incubated for 48 h, followed by analysis using BrdU incorporation assay (Roche, Basel, Switzerland), according to the manufacturer's instructions.

### *RNA extraction, cDNA synthesis, and quantitative real-time polymerase chain reaction analysis*

For the experiments, KFBs were seeded into each well of 6-well flat bottom plates and cultured in culture medium with 10% FBS. After 24 h, FAs were added and the cells were incubated for 48 h. Then, the cells were processed for total RNA isolation using TRIzol (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions and reverse transcribed to yield a single-stranded cDNA, using iScript cDNA synthesis kits (Bio-Rad, Hercules, CA) according to the manufacturer's instructions. The cDNAs were used for the subsequent quantitative real-time polymerase chain reaction (PCR) analysis, using SYBR Premix Ex Taq II (Takara Bio, Otsu, Japan) with each primer (Table 1).<sup>16,23-26</sup> The PCR reactions were run on iCycler IQ (Bio-Rad, Hercules, CA) for 40 cycles at 95°C for 30 s, at an annealing temperature (Table 1) for 30 s, and at 72.0°C for 30 s. Post-polymerase chain reaction melting curves were confirmed by the specificities of single-target amplification, and the relative expressions of each gene were calculated in duplicate based on glyceraldehyde-3-phosphate dehydrogenase (GAPDH) expression.

### *Western blotting*

KFBs were seeded into each well of 6-well flat bottom plates and cultured in culture medium with 10% FBS. After 24 h, FAs were added and the plates were incubated for 48 h. After preparation of the KFBs in 1.5 mL tubes, they were suspended to 100  $\mu$ L of Pro-Prep (iNtRON, Gyeonggi-do, Korea), according to the manufacturer's instructions. Five microliters of the cell supernatants was used to measure the protein concentration, using Lowry's method (RC DC Protein Assay Kit, Bio-Rad, Hercules, CA). Western blotting was performed as described previously, using primary antibodies against  $\alpha$ -SMA (1:400; Sigma), acetyl-histone H3 (1:1000; Cell Signaling Technology Inc., Danvers, CO), PPAR $\gamma$  (1:800; Santa Cruz Biotechnology Inc., Santa Cruz, CA), GAPDH (1:40000; Sigma), and appropriate horseradish peroxidase-conjugated secondary antibody.<sup>27</sup> Densitometric results were analyzed using the Image J software (National Institutes of Health, Bethesda, MD).

### *Measurement of PGE<sub>2</sub> levels in culture supernatants*

PGE<sub>2</sub> concentrations were measured using enzyme immunoassay kits (R&D Systems, Minneapolis, MN), according to the

manufacturer’s protocol, and an enzyme-linked immunosorbent assay reader (Benchmark Microplate Reader, Bio-Rad, Tokyo, Japan).

*Immunofluorescence staining*

The morphological features of F-actin and the nuclei in KFBs were analyzed by immunofluorescence staining, as described previously, using phallotoxins (1:40; Invitrogen, Carlsbad, CA) and 4',6-diamidino-2-phenylindole (1:1000; Dojin, Kumamoto, Japan). Immunofluorescent staining patterns were observed with a BX50 fluorescence microscope at ×200 magnification (Olympus, Tokyo, Japan) and recorded with a digital camera (EOS Kiss X4, Canon, Tokyo, Japan). Apoptotic cells were identified by nucleus condensation and fragmentation, and nuclei from 10 random fields of each coverslip were examined at ×100 magnification.

*Statistical analysis*

Data from independent experiments were used to calculate the mean ± SD values. Differences were considered significant if *p* < 0.05, as determined by the Tukey-Kramer post hoc test.

**RESULTS**

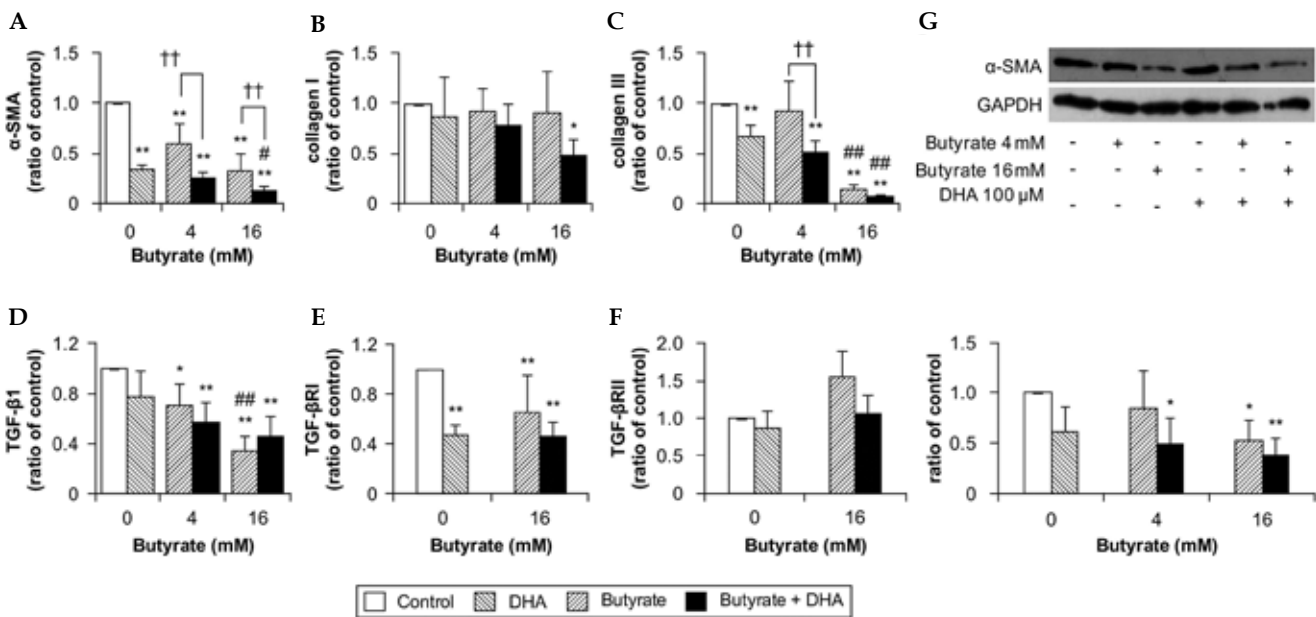
**Profibrotic factor and TGF-β1 signal expressions**

We evaluated the effects of butyrate and DHA on profibrotic factors in KFBs from chest. As for α-SMA expression, butyrate or DHA inhibited α-SMA mRNA expression similarly (*p* < 0.01; Figure 1). The butyrate/DHA combination augmented the inhibitory effect of butyrate (13.1% of the control; *p* < 0.01). This change in mRNA level was obvious in the protein level. As for collagen expression, the butyrate or DHA treatment inhibited collagen III expression (14.2% or 68.2%, respectively; *p* < 0.01). The butyrate/DHA combination increased the inhibitory effect, resulting in additional inhibition of collagen III (7.5% of the control) and collagen I expressions (49.6% of the control; *p* < 0.05). Since butyrate and DHA exerted the similar effects on these profibrotic factors in KFBs from earlobe, the following experiments were conducted in KFBs from chest.

To further characterize the inhibition of profibrotic factors, we investigated the TGF-β1 signal mRNA expression (Figure 1). Butyrate

**TABLE 1: Primers used for real-time polymerase chain reaction**

Gene	Forward (5'-3')	Reverse (5'-3')	Annealing
GAPDH	CATCAAGAAGGTGGTGAAGC	CCTCCCCAGCAAGAATGTCT	62.5 °C
α-SMA	CGTGGGTGACGAAGCACAG	GGTGGGATGCTCTTCAGGG	62.5 °C
TGF-β1	GGGACTATCCACCTGCAAGA	CCTCCTGGCGTAGTAGTCG	62.5 °C
collagen I	GTGCTAAAGGTGCCAATGGT	ACCAGGTTACACCGCTGTAC	57.5 °C
collagen III	TATCGAACCGCAAGGCTGTGAGA	GGCCAACGTCCACACCAAATTCTT	65.8 °C
COX-1	ATGATGGGCCTGCTGTGGA	CCAACACTCACCATGCCAAAC	61.5 °C
COX-2	TCCCTGAGCATCTACGGTTTG	AACTGCTCATACCCCATTC	61.7 °C
TGF-βRI	GGTCTTGCCCATCTTCACAT	TCTGTGGCTGAATCATGTCT	56.0 °C
TGF-βRII	TTTGGGCTTTCCTGCGTCT	TCTGGAGCCATGTATCTTGCAGTTC	64.5 °C



KFBs were exposed for 48 h to the indicated concentrations of butyrate with/without 100 μM DHA. The (a) α-SMA, (b) collagen I, (c) collagen III, (d) TGF-β1, (e) TGF-βRI, and (f) TGF-βRII mRNA expressions were analyzed by real-time polymerase chain reaction. (g) The α-SMA and GAPDH protein expressions were analyzed by western blotting. Results from a representative experiment are shown. Similar results were obtained from 6 independent experiments. The graph shows the α-SMA/GAPDH ratio. Data from 6 independent experiments were used to calculate mean ± SD (\**p* < 0.05 and \*\**p* < 0.01, vs. the control; #*p* < 0.05 and ##*p* < 0.01, vs. DHA; †*p* < 0.05 and ††*p* < 0.01, butyrate vs. butyrate + DHA).

**FIGURE 1: Effects of butyrate with/without DHA on the profibrotic factors and TGF-β1 signaling expressions in KFBs**

inhibited the TGF-β1 (70.4% of the control and 34.1% at 4 and 16 mM) and TGF-βRI expressions (65.8% at 16 mM;  $p < 0.01$ ). DHA inhibited TGF-βRI expression (47.2%;  $p < 0.01$ ). However, butyrate or DHA did not affect the TGF-βRII expression. The effect of the butyrate/DHA combination was similar to that of the monotherapy in each FA.

*KFB growth, survival, and apoptosis*

Butyrate or the butyrate/DHA combination reduced the number of cells in the control to 51.2% or 48.8% with 4 mM butyrate and 40.8% or 43.2% with 16 mM butyrate ( $p < 0.01$ ), respectively, with no change in cell viability (Figure 2). These changes in cell number were accompanied by those obtained in the 5-bromo-2'-deoxyuridine (BrdU) incorporation assay results, that is, 23.3% or 23.6% in 4 mM butyrate and 9.4% or 10.6% in 16 mM butyrate, respectively ( $p < 0.01$ ). DHA indicated no effect.

DNA fragmentation was clearly observed after 96 h of treatment with 16 mM butyrate ( $p < 0.01$ ) but was not detected after 48 h of treatment (data not shown), accompanied with high cellular viability. The butyrate/DHA combination-induced DNA fragmentation slightly augmented the effect of butyrate.

**Alteration of histone acetylation and PPARγ expression in KFBs**

Butyrate induced histone H3 acetylation dose-dependently by 3.7- and 5.5-fold at 4 and 16 mM, respectively ( $p < 0.01$ ), indicating inhibition of HDAC activity. In contrast, DHA did not alter this activity (Figure 3). The result of the butyrate/DHA combination was similar to that of the butyrate-only treatment.

To determine the mechanism of action of DHA, we also evaluated the PPARγ expression (Figure 3). Butyrate or DHA did not induce a significant PPARγ expression at 48 h of treatment.

*F-Actin arrangement*

Butyrate at 16 mM or DHA at 100 μM decreased stress fiber formation, with butyrate having a stronger effect than DHA (Figure 4). The butyrate/DHA combination also strongly disrupted the stress fibers in the cytoplasm after 48 h of treatment.

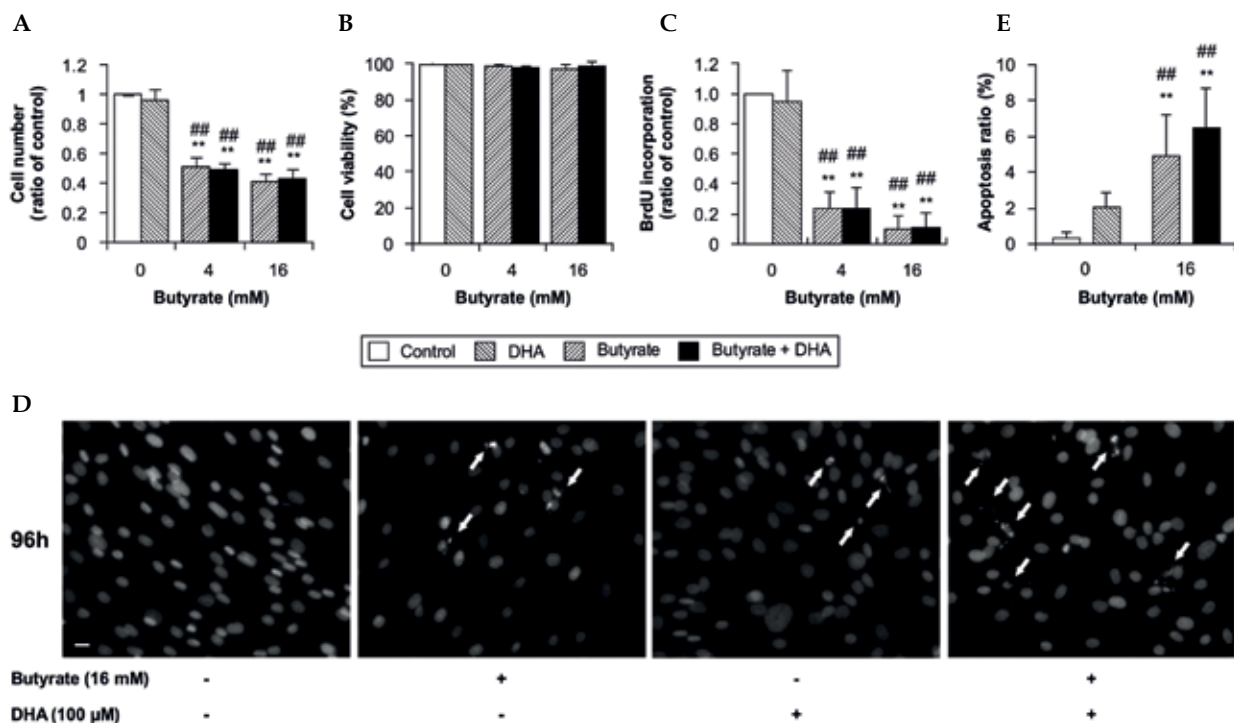
*PGE<sub>2</sub> synthesis*

Butyrate increased PGE<sub>2</sub> secretion by 4.6-fold of the control at 16 mM ( $p < 0.01$ ; Figure 5), whereas DHA did not. The result of the butyrate/DHA combination was similar to that of the butyrate-only treatment.

Butyrate significantly increased the COX-1 expression level to 174.9% of the control ( $p < 0.05$ ). Butyrate or DHA slightly suppressed the COX-2 expression to 70.2% or 82.6%, respectively, but not significantly. The butyrate/DHA combination suppressed the COX-2 expression to 61.9% ( $p < 0.05$ ).

**DISCUSSION**

This is the first report to indicate the antifibrogenic effects of butyrate and DHA on KFBs. This study presents 3 novel findings. First, butyrate inhibited cell proliferation and α-SMA, collagen III, TGF-β1, and TGF-βRI expressions and induced apoptosis, PGE<sub>2</sub> secretion, COX-1 expression, stress fiber disruption, and histone acetylation. Second, DHA inhibited α-SMA, collagen III, and



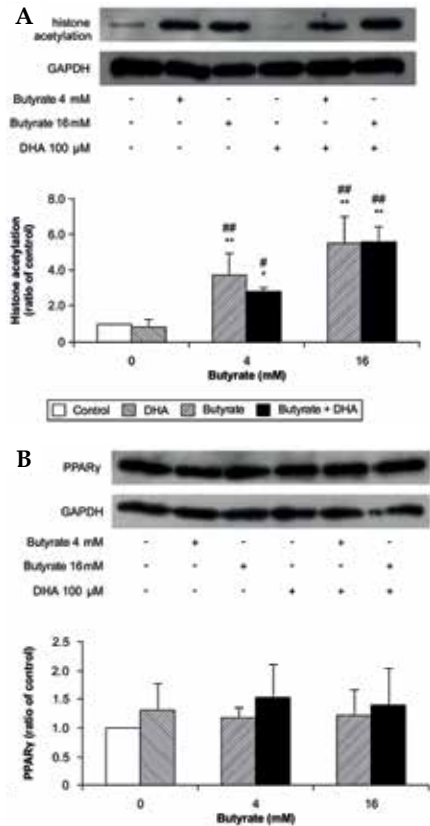
KFBs were exposed for 48 h to the indicated butyrate concentrations with/without 100 μM DHA. (a) The cell number and (b) viability were determined by trypan blue staining. (c) Proliferation was assessed using the BrdU incorporation assay. Data from 6 (cell number and viability) and 10 (BrdU assay) separate cultures were used to calculate mean ± SD. (d) White arrows indicate DNA fragmentation. KFBs were exposed for 96 h to 16 mM butyrate with/without 100 μM DHA. The micrographs are representative of all the cell cultures. (e) DNA fragmentation from 10 random fields of each coverslip was used to calculate mean ± SD (\*\* $p < 0.01$ , vs. the control cultures; ## $p < 0.01$ , vs. DHA). Scale bar, 20 μm

**FIGURE 2:** Effects of butyrate with/without DHA on KFB proliferation, survival, and apoptosis

TGF-βRI expressions but did not alter PPARγ expression. Third, the butyrate/DHA combination augmented the inhibitory effects on α-SMA, collagen I, collagen III, TGF-β1, and TGF-βRI expressions; inhibited cell proliferation; induced apoptosis; and strongly dis-

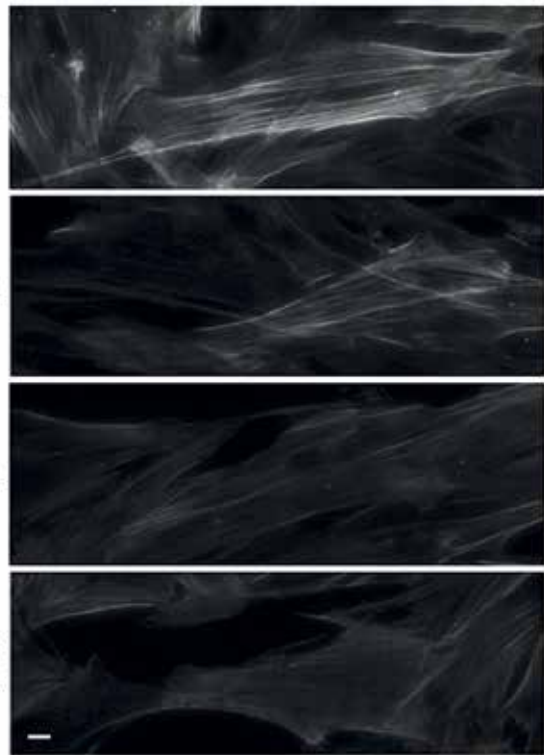
rupted stress fibers. These findings suggest the therapeutic effect of the butyrate/DHA combination on the fibrogenesis of keloids.

The present finding of strong inhibition of cell proliferation without cell viability change by butyrate is in agreement with that of our previous report using NFBs and other reports using several normal fibroblasts.<sup>16,28,29</sup> This agreement suggests the efficacy of butyrate for not only normal fibroblasts but also activated fibroblasts in fibroproliferative disorders.



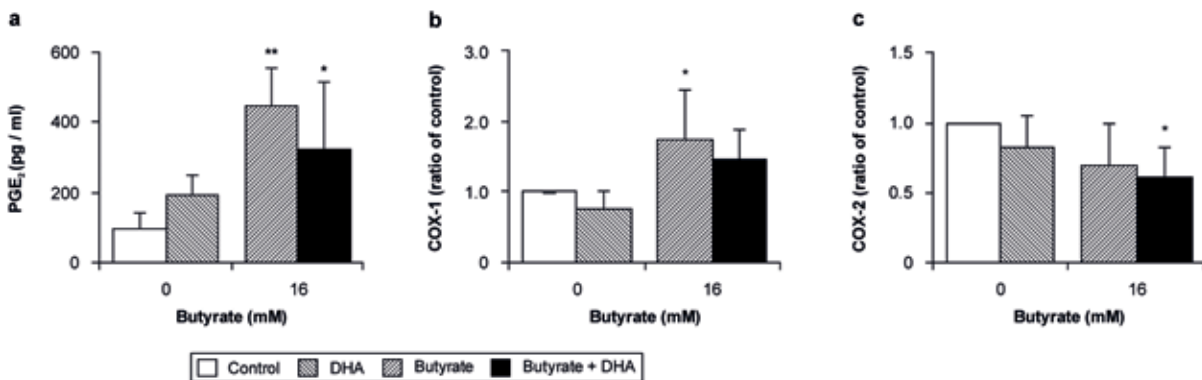
KFBs were exposed for 48 h to the indicated concentrations of butyrate with 100 μM DHA. The protein expressions of (a) histone acetylation and (b) PPARγ were analyzed using western blotting. Results from a representative experiment are shown. Similar results were obtained from 6 independent experiments. The graphs show the histone acetylation/GAPDH and PPARγ/GAPDH ratios. Data from 6 independent experiments were used to calculate the mean ± SD values (\*p < 0.05 and \*\*p < 0.01, vs. the control)

FIGURE 3 : Effects of butyrate with/without DHA on histone acetylation and PPARγ expression in KFBs



KFBs were exposed to DHA (100 μM), butyrate (16 mM), and butyrate with DHA (16 and 100 μM, respectively) for 48 h. The morphological organizations of F-actin and the nuclei in KFBs were analyzed by immunofluorescence staining with phallothoxins (F-actin) and 4',6-diamidino-2-phenylindole (nuclei). The micrographs are representative of all the cell cultures. Scale bar, 10 μm

FIGURE 4 : Disruption of stress fibers by butyrate and DHA in KFBs



KFBs were exposed for 48 h to the indicated concentrations of butyrate with 100 μM DHA. (a) PGE2 concentrations in the medium were then measured. The mRNA expressions of (b) COX-1 and (c) COX-2 were analyzed by real-time polymerase chain reaction. Data from 6 independent experiments were used to calculate mean ± SD (\*p < 0.05 and \*\*p < 0.01, vs. the control; #p < 0.05 and ##p < 0.01, vs. DHA).

FIGURE 5 : Effects of butyrate with/without DHA on PGE2 secretion, and COX-1 and COX-2 mRNA expressions in KFBs

The butyrate-induced histone acetylation is in agreement with a number of reports indicating antifibrogenic effects with the mechanism of cell cycle arrest at the G<sub>1</sub>/S phase and changes in a number of cell cycle regulatory gene expressions in rat embryonic fibroblasts.<sup>13,30,31</sup> Considering our results and those of the other reports, we suggest that the mechanism of the antiproliferative activity in KFBs could be due to the butyrate-induced histone acetylation. Our result of DNA fragmentation after 96 h of treatment with butyrate coincides with the report using porcine fetal fibroblasts.<sup>29</sup> The stronger induction of apoptosis by the butyrate/DHA combination than that by monotherapy is in agreement with reports indicating the mechanism of synergistic induction of apoptosis in colonocytes.<sup>32</sup> From those reports, we suspect that the butyrate and DHA combination synergistically enhances apoptosis via an additional intrinsic mitochondrial pathway to a FAs-mediated extrinsic pathway reported to be activated in colonic cells by butyrate.<sup>33</sup> Further studies to elucidate this mechanism in KFBs are required.

The inhibition of collagen III and  $\alpha$ -SMA expressions by butyrate or DHA is in agreement with our previous report on NFBs.<sup>16</sup> The butyrate/DHA combination augmented the antifibrogenic effects, resulting in additional suppression of  $\alpha$ -SMA, collagen I, and collagen III expressions, which was stronger than that by butyrate monotherapy. Although 24-h FA treatment of KFBs did not show antifibrogenic effects in preliminary experiment different from those of NFBs,<sup>16</sup> 48 h FA treatment showed strong antifibrogenic effects in KFBs, therefore suggesting that the long term FA treatment in fibroproliferative disorders requires cell activity inhibition.

In terms of TGF- $\beta$ 1 signaling, the inhibition of TGF- $\beta$ 1 and TGF- $\beta$ RI mRNA expressions by butyrate indicates that butyrate could inhibit TGF- $\beta$ 1 signaling, followed by inhibitions in  $\alpha$ -SMA and collagen expressions. Meanwhile, stress fiber disruption by butyrate as well as histone acetylation in the present study is in agreement with the report on human lung cell line demonstrating stress fiber disruption by histone acetylation using the phenylbutyrate-based HDAC inhibitor.<sup>34</sup> Furthermore, the inhibition of  $\alpha$ -SMA expression accompanied with stress fiber disruption by butyrate in the present study is in agreement with our previous report on NFBs, and stress fiber formation is reported to upregulate  $\alpha$ -SMA expression in fibroblasts.<sup>16,35</sup> Therefore, stress fiber regulation would be one of the mechanisms in antifibrogenic effects by butyrate in addition to inhibition of TGF- $\beta$ 1 signaling.

DHA induced neither histone acetylation nor drastic stress fiber disruption, as observed in the butyrate treatment in this study. Therefore, the mechanism of the antifibrogenic effects of DHA differs from that of butyrate. At first, we hypothesized that the anti-

fibrogenic mechanism of DHA was via the upregulation of PPAR expression. However, in the present study, DHA did not alter the PPAR $\gamma$  expression at 48 h of treatment, in agreement with Ghosh's study using naturally occurring and synthetic pharmacological PPAR $\gamma$  ligands, showing no change in total cellular PPAR $\gamma$  level but an increase in PPAR $\gamma$  nuclear levels and activated PPAR $\gamma$  resulting in antifibrogenic effects.<sup>36</sup> Therefore, it can be speculated that the antifibrogenic effects of DHA is due to the change in PPAR $\gamma$  nuclear levels or PPAR $\gamma$  activation. However, this requires confirmation in further experiments with KFBs.

The finding that PGE<sub>2</sub> secretion and COX-1 expression increased with the butyrate treatment in KFBs is in agreement with that of our previous report on human peripheral blood mononuclear cells.<sup>12</sup> Furthermore, Taniura *et al.* reported that trichostatin A, a potent HDAC inhibitor increased PGE<sub>2</sub> secretion with upregulation of COX-1 expression in human astrocyte cells.<sup>37</sup> Altogether, their results and our results indicate that the mechanism of increased PGE<sub>2</sub> secretion in the present study could be due to increasing COX-1 expression by the butyrate-induced HDAC inhibitor activity. PGE<sub>2</sub> was reported to inhibit collagen synthesis and  $\alpha$ -SMA in KFBs activated by TGF- $\beta$ 1 and fibroblasts of idiopathic pulmonary fibrosis.<sup>38,39</sup> Therefore, we suggest that the increase in PGE<sub>2</sub> secretion by butyrate could be an underlying mechanism of profibrotic factor inhibition. Although the effect of the butyrate/DHA combination on PGE<sub>2</sub> secretion was similar to that of the butyrate monotherapy, the butyrate/DHA combination inhibited COX-2 expression, suggesting the importance to control inflammatory signaling in keloid tissues.<sup>40</sup> Considering this report, we suggest that an increase in PGE<sub>2</sub> secretion with downregulation of COX-2 expression could be beneficial for keloid therapies because of the anti-inflammatory and antifibrogenic effects. However, further studies to elucidate the anti-inflammatory effects of the butyrate/DHA combination are needed.

Activation in keloid tissue is affected not only by fibroblasts but also by epithelial cells and immune cells such as keratinocytes and neutrophils.<sup>41</sup> Therefore, evaluation of the effects of FAs on activated fibroblasts by cytokines, such as TGF- $\beta$ 1 and IL-1 $\beta$ , released from these cells is required to elucidate the effects on keloid tissues. Similarly, evaluation of the butyrate/DHA combination in an *in vivo* study is also required; however, a human model of keloid has not been established yet.

## CONCLUSIONS

We demonstrated the antifibrogenic effects of butyrate and DHA on KFBs. These findings could contribute to the development of novel therapy for dermal fibroproliferative disorders. □

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