• Basic Research •

Suppression of fibrosis in human pterygium fibroblasts by butyrate and phenylbutyrate

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Abstract

• AIM: To evaluate the antifibrogenic effects of butyrate or phenylbutyrate, a chemical derivative of butyrate, in human pterygium fibroblasts.

• METHODS: Human pterygium fibroblasts obtained from patient pterygium tissue were treated with butyrate or phenylbutyrate for 48h. Expression of α-smooth muscle actin, collagen I, collagen III and matrix metalloproteinase-1 mRNA was measured by quantitative real-time reverse transcription polymerase chain reaction, and acetylated histone was evaluated by Western blotting.

• RESULTS: Butyrate inhibited α-smooth muscle actin, type III collagen and matrix metalloproteinase-1 expressions, and phenylbutyrate inhibited types I and III collagen and matrix metalloproteinase-1 expressions without changing cell viability as well as both of these increased histone acetylation. These results suggested that butyrate and phenylbutyrate suppress fibrosis through a mechanism involving histone deacetylase inhibitor.

• CONCLUSION: This indicates that butyrate or phenylbutyrate have antifibrogenic effects in human pterygium fibroblasts and could be novel types of prophylactic and/or therapeutic drugs for pterygium, especially phenylbutyrate, which does not have the unpleasant smell associated with butyrate.

• KEYWORDS: butyrate; phenylbutyrate; pterygium; fibroblasts; antifibrogenic effect

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INTRODUCTION

Pterygium is a triangular-shaped overgrowth of the fibrovascular conjunctiva onto the nasal or temporal cornea, caused mainly by chronic exposure to ultraviolet rays. It may cause ocular irritation, cosmetic problems, astigmatism and visual impairment. Although surgical removal is performed for these symptomatic cases[1-2], post-surgical recurrence rates after excision of the pterygium have been reported to be very high. Several intra- and post-operative treatments, including mitomycin C, 5-fluorouracil and corticosteroids, have been recommended for the prevention of postoperative recurrence of pterygium[2]. However, despite these treatments, the recurrence of pterygium and/or occasional severe complications may occur. Therefore, safer and securer treatments for the prevention of the recurrence of pterygium are strongly desirable.

Aberrant extracellular matrix (ECM) remodeling appears to be a major feature of pterygium, as evidenced by previous studies indicating that ECM genes including fibronectin, collagen and versican are upregulated in pterygium[3-4]. In particular, matrix metalloproteinases (MMPs), a family of structurally related zinc-dependent ECM-degrading proteinases, are elevated in pterygium[5]. In the process of abnormal cell proliferation and angiogenesis generation in primary and recurrent pterygium, modification or degradation of ECM may be related to MMPs. It has been reported that altered limbal basal epithelial cells may cause activation of fibroblasts at the head of the pterygium, leading to degradation of Bowman's layer as a result of the production of MMP-1 derived from fibroblasts and further invasion of pterygium into the cornea[6]. Regarding the proliferative gain of function of fibroblasts, myofibroblasts express α-smooth muscle actin (α-SMA), and its expression in pterygium has been reported in a previous study[7].

Butyrate, a predominant short-chain fatty acid, is one of the end products of anaerobic bacterial fermentation of dietary fibers in the colon, and has histone deacetylase (HDAC) inhibitor activity[8]. Butyrate or other HDAC inhibitors have antifibrogenic effects that suppress collagen synthesis, α-SMA expression and increase of cell number in various organs, such as pancreas or lung[9-10]. We have previously reported that the antifibrogenic effects of butyrate are stronger than propionate in human dermal fibroblasts[11]. However, these effects of butyrate in human pterygium fibroblasts (HPFs) have not been investigated. Because butyrate has a characteristic unpleasant smell, it is considered that it might be unsuitable for ocular administration in humans.

Phenylbutyrate (PB), a chemical derivative of butyrate without the unpleasant smell, is a non-toxic pharmacological compound that functions as a weaker HDAC inhibitor than butyrate. The Food and Drug Administration has approved its clinical use in the United States in patients with urea cycle disorders and hyperammonemia[12]. PB has also been used in clinics to treat β-thalassemia, sickle cell anemia and cancer[13-14]. It has been reported that PB decreases the expression of collagen I through histone acetylation in fibroblast from human lung[10]. However, these effects of PB in HPFs have not been investigated. It has also been reported that topical ocular PB administration shows beneficial effects in improving glaucoma in a mouse model without causing eye abnormalities[15].

In the present study, we investigated the antifibrogenic effects of butyrate and PB in HPFs by measuring profibrotic factors and MMP expression, and the underlying mechanism involving histone acetylation.

SUBJECTS AND METHODS

Cell Culture HPFs were cultivated from pterygium obtained from two patients during surgical removal, and the effects of butyrate and PB were evaluated in HPFs from each patient. Patient 1 was a woman aged 68 with a primary pterygium, and patient 2 was a man aged 78 with recurrent pterygium. The protocol for tissue collection and analysis was approved by the ethics board of Kobe University Graduate School and followed the Declaration of Helsinki. Written informed consents were obtained from all of the participants. After surgical excision, subconjunctival connective tissue of pterygium was propagated in Dulbecco's modified Eagle medium (DMEM; Wako, Osaka, Japan) supplemented with 10% fetal bovine serum (FBS; Nichirei, Tokyo, Japan), 50 U/mL penicillin and 50 μg/mL streptomycin (MP Biomedicals, Illkirch, France). The culture medium was changed every 2 or 3d until approximately 80% confluence was reached. The cells were passaged by incubation at 37℃ with 0.125% trypsin-EDTA, and plated in culture dishes. Cells at passages 3 to 7 were used for experiments, and we confirmed the fast growth of these cells. Four independent experiments were performed for all analyses in HPFs from each patient. The trial and additional data collection on the cause of visual loss were approved by the relevant local research ethics committees.

Trypan Blue Staining For experimental treatments, the cells were seeded into six-well plates (Iwaki; Tokyo, Japan) at a concentration of 2.8×10^5 cells/well and incubated for 24h. The cells were then cultured in DMEM with 0 (control), 1, 4 or 16 mmol/L sodium butyrate (Sigma, St. Louis, MO, USA) or sodium PB (Sigma) for 48h. Trypan blue staining was used to calculate cell viability.

Quantitative Real-time Polymerase Chain Reaction HPFs were cultured in six-well plate using DMEM supplemented with 10% fetal bovine serum. After 24h, the content of serum in the medium was reduced to 0.1% for 48h to render the cells quiescent. Sodium butyrate and sodium PB were added and the HPFs were incubated for 48h. Then, they were processed for total RNA isolation using TRIzol (Invitrogen, Carlsbad, CA, USA) and reverse transcribed to yield a single-stranded cDNA, with iScript cDNA synthesis kits (Bio-Rad, Hercules, CA, USA). The expressions of collagen I, collagen III, α-SMA and MMP-1 were detected by means of quantitative real-time polymerase chain reaction (PCR) analysis, using SYBR Premix Ex Taq II (Takara Bio, Otsu, Japan) with each primer (Table 1). PCRs were run on iCycler IQ (Bio-Rad, Hercules, CA, USA) for 40 cycles at 95℃ for 30s, at an annealing temperature (Table 1) for 30s, and at 72.0℃ for 30s. Post-PCR melting curves were confirmed by the specificities of single-target amplification, and the relative expressions of each gene were calculated based on glyceraldehyde-3-phosphate dehydrogenase (GAPDH) expression in duplicate.

Western Blotting HPFs were cultured in six-well plate using DMEM supplemented with 10% FBS. After 24h, the content of serum in the medium was reduced to 0.1% for 48h to render the cells quiescent. Sodium butyrate and sodium PB were added and the HPFs were incubated for 48h. The HPFs were prepared in 1.5-mL tubes and then suspended in 100 μL of ProPrep (iNtRON, Gyeonggi-do, Korea). Aliquots (5 μL) of the cell supernatants were used to measure the protein concentration using Lowry's method (RC DC Protein Assay Kit: Bio-Rad, Hercules, CA, USA). Western blotting was performed as described previously[16] using primary antibodies against acetyl-histone H3 (1:1000; Cell Signaling Technology Inc., Danvers, MA, USA) and GAPDH (1:10000; Sigma), and appropriate horseradish peroxidase-conjugated secondary antibody. Densitometric results were analyzed using Image J software (National Institutes of Health, Bethesda, MD, USA).

Statistical Analysis The data are expressed as the mean± standard error (SE). Differences were considered statistically significant if the P value was <0.05, as determined using the Tukey-Kramer post hoc test.

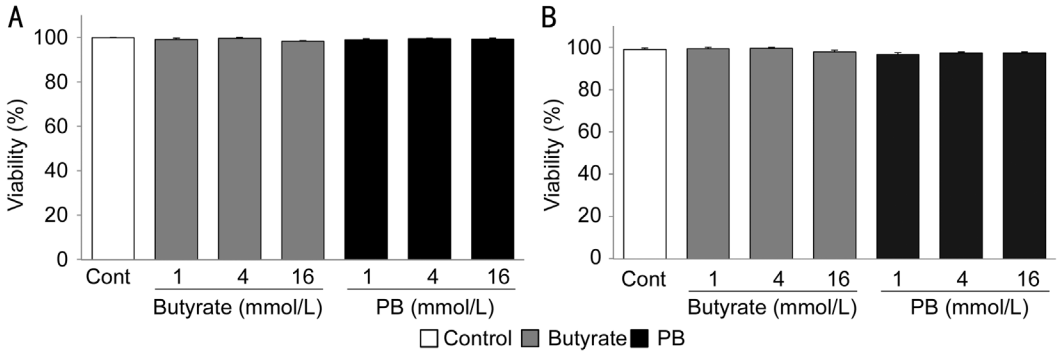

Figure 1 Effects of butyrate or PB on viability of HPFs HPFs of (A) patient 1 and (B) patient 2 were exposed to the indicated concentrations of PB or butyrate for 48h. Cell viability were determined by trypan blue staining. Mean value and standard error (SE) were calculated from data of four separate cultures.

Table 1 Primers used in real-time PCR

Gene	Forward (5'-3')	Reverse (5'-3')	Annealing (℃)
GAPDH	AAGGTGAAGGTCGGAGTCAA	AATGAAGGGGTCATTGATGG	62.5
Collagen I	GTGCTAAAGGTGCCAATGGT	ACCAGGTTCACCGCTGTTAC	57.5
Collagen III	TATCGAACACGCAAGGCTGTGAGA	GGCCAACGTCCACACCAAATTCTT	65.8
α-SMA	CGTGGGTGACGAAGCACAG	GGTGGGATGCTCTTCAGGG	62.5
MMP-1	CCTCGCTGGGAGCAAACA	TTGGCAAATCTGGCGTGTAAT	59.3

RESULTS

Cellular Toxicity The viability of HPFs from patient 1 (Figure 1A) and patient 2 (Figure 1B) treated with butyrate or PB was >96%, and there were no significant differences in cell viability among all treatments.

Collagen I Expression in Human Pterygium Fibroblasts Treated with Butyrate or Phenylbutyrate To investigate the effective treatment time for butyrate or PB, we assessed collagen I expression in HPFs of patient 1 treated with these compounds for 24 or 48h. Butyrate did not change collagen I expression at both 24 and 48h (Figure 2A, 2B). PB at a concentration of 16 mmol/L significantly inhibited collagen I expression at both 24 and 48h (P<0.05; Figure 2C, 2D); however, PB at a concentration of 4 mmol/L significantly inhibited collagen I expression only at 48h (P<0.05; Figure 2D). Therefore, the following experiments were performed involving treatment with butyrate or PB for 48h.

Effect of Butyrate on Profibrotic Factors and Matrix Metalloproteinases Expressions Regarding mRNA expression of profibrotic factors, butyrate at concentrations of 4 and 16 mmol/L significantly inhibited α-SMA expression by 50% of the control level (P<0.01) and also collagen III mRNA expression in HPFs of each patient (Figure 3A, 3C, 3E and 3G). In particular, butyrate at a concentration of 16 mmol/L strongly suppressed α-SMA (patient 1, 22.7%; patient 2, 25.7%; P<0.01) and collagen III (patient 1, 7.7%; patient 2, 3.6%; P<0.01) expressions; however, the expression of collagen I was not suppressed (Figure 3B, 3F). Butyrate also significantly inhibited MMP-1 mRNA expression in HPFs of patient 1 (P<0.01; Figure 3D), but not in HPFs of patient 2 (Figure 3H).

Effect of Phenylbutyrate on Profibrotic Factors and Matrix Metalloproteinase Expressions PB significantly inhibited collagen I, collagen III and MMP-1 mRNA expressions in HPFs of each patient (P<0.01; Figure 4B-4D, 4F-4H). In particular, PB at a concentration of 16 mmol/L inhibited collagen I expression by 20% relative to the control in both HPFs. In contrast, PB did not inhibit α-SMA expression in both HPFs. These results suggest that butyrate and PB have antifibrogenic effects.

Alteration of Histone Acetylation To investigate the mechanism of antifibrogenic effect by butyrate and PB, acetyl-histone H3 protein was assessed. Butyrate induced acetylation of histone H3 in HPFs of each patient (P<0.01; Figure 5A, 5B, P<0.05; Figure 5D, 5E), indicating inhibition of HDAC activity. Although PB also induced acetylation of histone H3 in HPFs of each patient (Figure 5A, 5C, 5D, 5F), only the change in HPFs of patient 2 was statistically significant (P<0.01). The acetylation of histone H3 was more strongly induced by butyrate than PB in both HPFs, indicating butyrate is a more effective HDAC inhibitor than PB.

DISCUSSION

This is the first report to demonstrate the antifibrogenic effects of butyrate or PB in HPFs obtained from patients undergoing pterygium surgery; there were three principle findings. First, butyrate or PB did not affect the cell viability of HPFs from each patient. Second, butyrate inhibited α-SMA, collagen III and MMP-1 expression. Third, PB inhibited collagen I, collagen

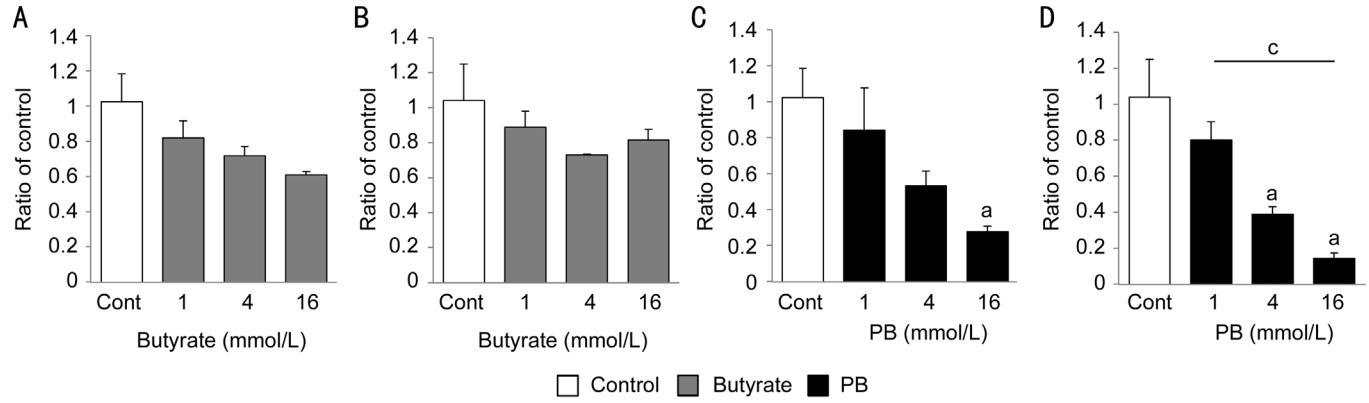

Figure 2 Collagen I expression in HPFs treated with butyrate or PB for 24 or 48h HPFs of patient 1 were exposed to the indicated concentrations of PB or butyrate for 24 or 48h. mRNA expression of collagen I at (A) 24h, (B) 48h treated with butyrate, (C) 24h and (D) 48h treated with PB were analyzed using real-time PCR analysis. Mean value and SE were calculated from data of four separate cultures. [a]$P<0.05$ *vs* control; [c]$P<0.05$ (Tukey-Kramer).

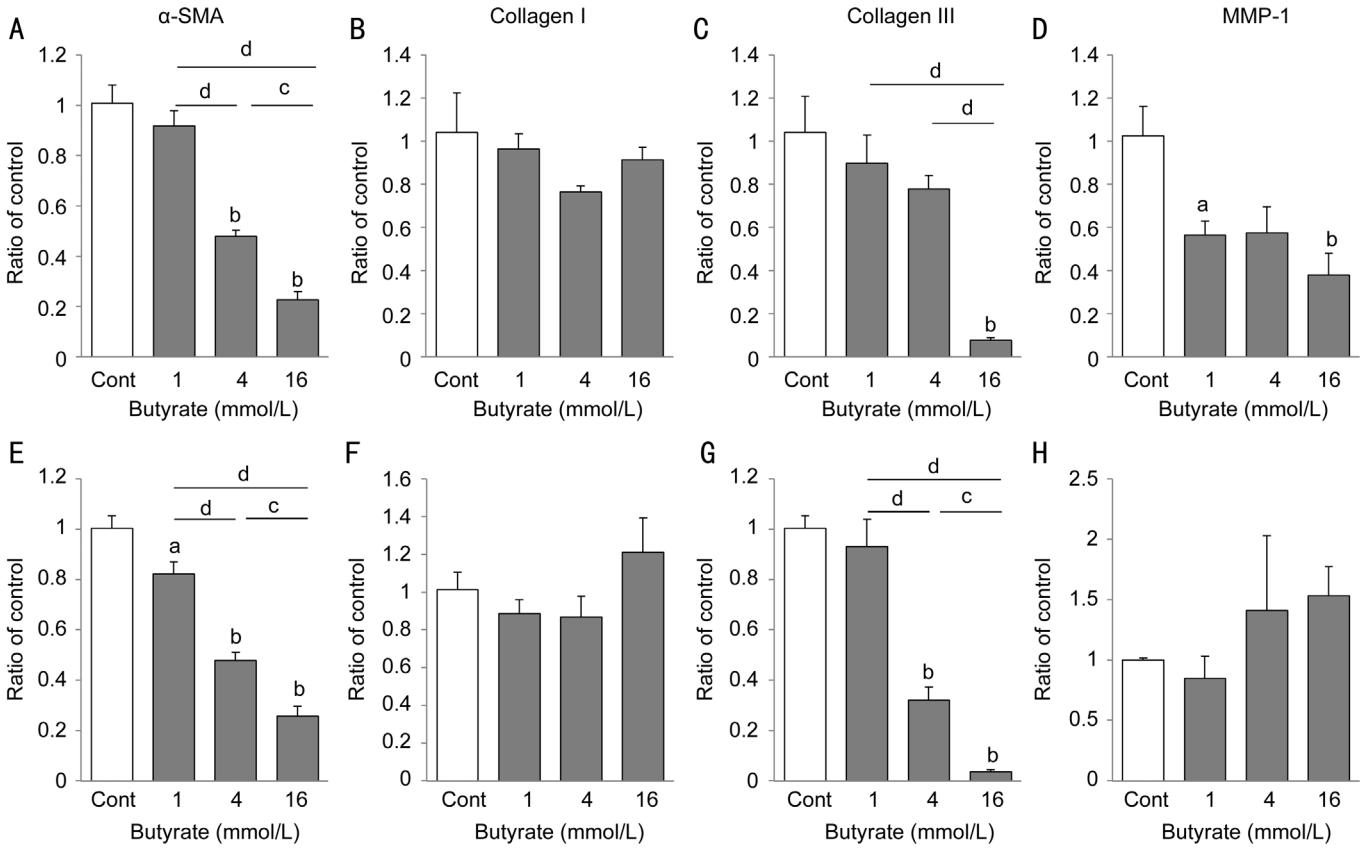

Figure 3 Effects of butyrate on the expression of profibrotic factors and MMP in HPFs HPFs from (A-D) patient 1 and (E-H) patient 2 were exposed to the indicated concentrations of butyrate for 48h. mRNA expression of (A, E) α-SMA, (B, F) collagen I, (C, G) collagen III, (D, H) MMP-1 were analyzed using real-time PCR analysis. Mean value and SE were calculated from data of four separate cultures. [a]$P<0.05$; [b]$P<0.01$ *vs* control; [c]$P<0.05$; [d]$P<0.01$ (Tukey-Kramer).

III and MMP-1 expression. PB and butyrate were found to inhibit profibrotic factors and MMP; however, PB inhibited collagen III expression at lower concentrations than butyrate. In particular, PB at a concentration of 1 mmol/L, the lowest concentration in this study, inhibited collagen III expression in HPFs of patient 2. These findings suggest that butyrate or PB have therapeutic inhibitory effects on fibrogenesis and progression of pterygium, and that PB might be more suitable for clinical use than butyrate.

Expression of collagen I and collagen III was significantly inhibited in HPFs treated with PB at concentrations of 4 and 16 mmol/L for 48h, suggesting that PB has antifibrogenic effects. The inhibition of collagen I expression by PB is in agreement with a previous study involving human lung fibroblasts[10]. Rishikof *et al*[10] suggested that PB regulates collagen I expression by mechanisms that include stimulating

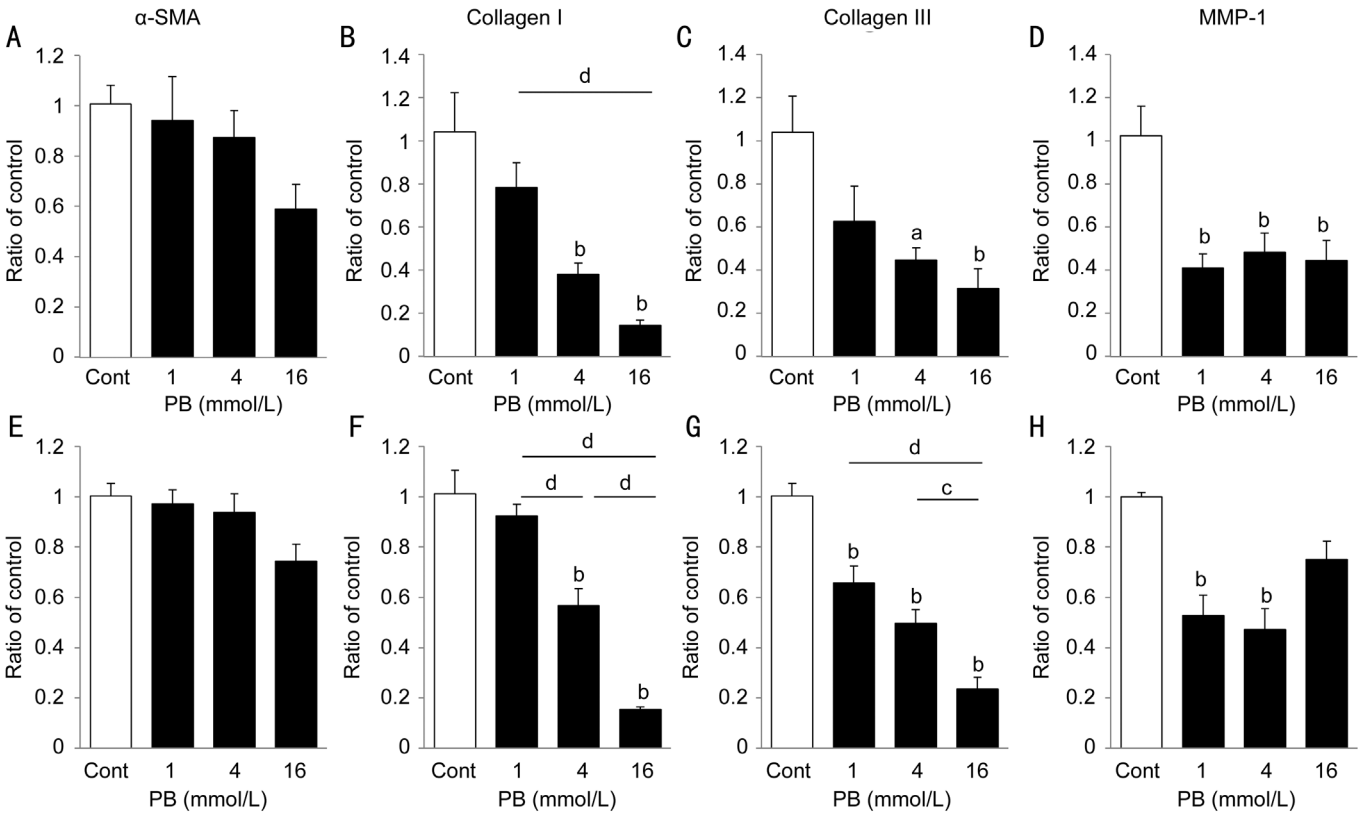

Figure 4 Effects of PB on the expression of profibrotic factors and MMP in HPFs HPFs from (A-D) patient 1 and (E-H) patient 2 were exposed to the indicated concentrations of PB for 48h. mRNA expressions of (A, E) α-SMA, (B, F) collagen I, (C, G) collagen III, (D, H) MMP-1 were analyzed using real-time PCR analysis. Mean value and SE were calculated from data of four separate cultures. [a]P<0.05; [b]P<0.01 *vs* control; [c]P<0.05, [d]P<0.01 (Tukey-Kramer).

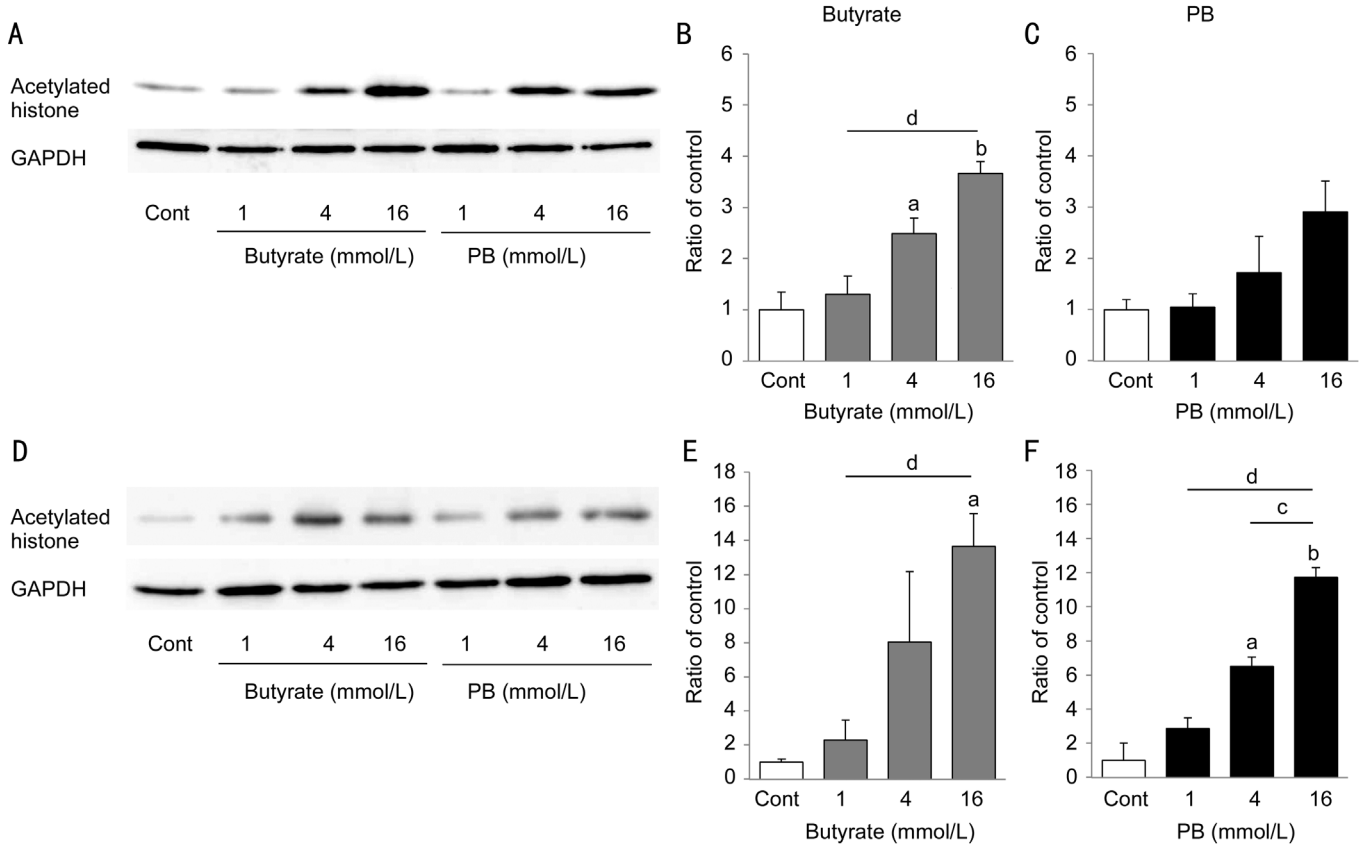

Figure 5 Effects of butyrate or PB on histone acetylation expression in HPFs HPFs of (A-C) patient 1 and (D-F) patient 2 were exposed to the indicated concentrations of PB or butyrate for 48h. Acetylated histone was analyzed by Western blotting. A, D: Results of a representative experiment are shown; B, C, E, F: Mean value and SE were calculated from data of four separate cultures. [a]P<0.05, [b]P<0.01 *vs* control; [c]P<0.05, [d]P<0.01 (Tukey-Kramer).

cAMP production and inhibiting HDAC activity. Although the well-known HDAC inhibitor butyrate did not change collagen I expression, the weaker HDAC inhibitor PB significantly inhibited collagen I expression. These results suggest that histone acetylation may be only partially responsible for the effect of PB on collagen I mRNA expression in HPFs, and that other mechanisms such as cAMP production might exist.

Expressions of α-SMA and collagen III were significantly inhibited in HPFs treated with butyrate at concentrations of 4 and 16 mmol/L for 48h. This inhibition of α-SMA and collagen III expressions by butyrate is in agreement with previous reports involving several mesenchymal cells[9]. Butyrate even at a concentration of 16 mmol/L did not inhibit collagen I expression; a similar response was observed in our previous study involving human dermal fibroblasts[11]. However, high doses of butyrate may inhibit collagen I expression in HPFs, because it has been reported that butyrate at a concentration of 20 mmol/L decreases collagen I mRNA levels in human lung fibroblasts[10].

Our study show that the HDAC inhibitors, butyrate and PB, suppress expression of profibrotic factors, indicate that the histone acetylation may have role of transcriptional regulator. It has been reported that HDAC inhibitors inhibit myofibroblastic differentiation and migration by inducing cell senescence in corneal stromal cells[17]. In addition, senescent hepatic stellate cells have been found to express reduced levels of ECM proteins, including collagens[18]. Therefore, the inhibition of profibrotic factor expression by butyrate or PB might be associated with the induction of senescence in HPFs. Krizhanovsky *et al*[19] have demonstrated that senescence of activated hepatic stellate cells limits liver fibrosis in a liver fibrosis model. Thus, senescence of HPFs might suppress fibrosis in pterygium tissue.

Inhibition of MMP-1 mRNA expression by butyrate or PB suggests that they could suppress the degradation of Bowman's layer and the infiltration of HPFs. It has been reported that HDAC inhibitors, including butyrate and trichostatin A, significantly reduce interleukin-1β-induced MMP-1 and MMP-3 expressions in human colonic subepithelial myofibroblasts[20]. Therefore, the inhibition of MMP-1 expression by butyrate or PB might be associated with their action regarding histone acetylation.

In the present study, we found the antifibrogenic effects of butyrate or PB at the level of mRNA. Inhibition of mRNA expression has been followed by decrease in protein expression in the previous studies[9-10]; however, evaluation of butyrate and PB effects in a protein level are required for sufficient assessment of their effects. In addition, the underlying mechanisms of antifibrogenic effect of butyrate and PB are still unclear; therefore, evaluation of the effects at further detailed studies including *in vivo* study is required.

We showed that PB, a derivative of butyrate without the unpleasant smell, has antifibrogenic effects at lower concentrations than butyrate. In addition, PB eye drops have been tested in a glaucoma mouse model, and did not cause eye abnormalities[15]. Thus, PB is considered to hold considerable promise in the treatment of pterygium as an eye drop formulation.

In summary, we demonstrated that butyrate and PB suppress fibrosis. These findings could contribute to the development of novel types of prophylactic and/or therapeutic drugs for the treatment of pterygium.

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