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Phosphodiesterase 7A inhibitor ASB16165 impairs proliferation of keratinocytes in vitro and in vivo

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ABSTRACT

Excessive proliferation of epidermal keratinocytes is a typical aspect of chronic skin diseases such as psoriasis. In the present study, the effect of phosphodiesterase 7A (PDE7A) inhibitor ASB16165 on proliferation of keratinocytes was investigated to examine the role of PDE7A in keratinocyte proliferation and the possible therapeutic relevance of PDE7A inhibition in psoriasis. Topical application of ASB16165 inhibited the increase of thickness of skin as well as epidermis in a skin inflammation model induced by repeated painting of 12-O-tetradecanoylphorbol-13-acetate (TPA) in a concentration-dependent manner. The ASB16165 treatment also suppressed the increase in the number of Ki67-positive keratinocytes in the model, showing the disturbance of keratinocyte proliferation by the treatment. In addition, both ASB16165 and dibutyryl cAMP significantly decreased the proliferation of human keratinocytes in vitro, suggesting that PDE7A participates in keratinocyte proliferation probably by controlling intracellular cAMP, while the contribution of other mechanism(s) is not completely denied. The findings in the present study indicate that the effect of ASB16165 on skin and epidermal hyperplasia in the TPA-induced skin inflammation is mediated, at least in part, by the inhibition of keratinocyte proliferation. The inhibitors for PDE7A including ASB16165 might be useful for the treatment of psoriasis.

1. Introduction

The epidermis, the surface layer of skin, plays an important role in the structural integrity and barrier formation and thereby participates in a defense system against an external environment (Eckert, 1989). Keratinocytes are the predominant cell type of the epidermis, constituting approximately 95% of the cell mass of the outer portion of the skin. Keratinocytes are also involved in immune and inflammatory reactions by secreting a variety of cytokines (Hauser et al., 1986; Kock et al., 1990; Mohamadzadeh et al., 1994). The proliferation of keratinocytes is known to be regulated by several soluble factors including cytokines in autocrine and paracrine manners (Piepkorn et al., 1998; Werner and Smola, 2001). Abnormal growth of keratinocytes is a prominent feature of psoriasis and has therefore been a target of the therapy of the disease (Lowes et al., 2007).

Phosphodiesterase 7 (PDE7) is a cAMP-specific PDE family (Michaeli et al., 1993) consisting of two members, PDE7A and PDE7B. PDE7A is highly expressed in the immune tissues including spleen and lymph nodes (Bloom and Beavo, 1996; Han et al., 1997), whereas PDE7B is undetectable in the leukocytes but abundant in brain, liver, heart, thyroid glands and skeletal muscles (Hetman et al., 2000). Interestingly, the inhibition of the activity or the expression of PDE7A blocks activation of T cells (Liu et al., 2006) as well as function/proliferation of preactivated T cells (Kadoshima-Yamaoka et al., 2009c) and cytotoxic T-lymphocytes (Kadoshima-Yamaoka et al., 2009b). In addition, an inhibitor for PDE7A, termed ASB16165, suppresses TNF-α production in the skin and ameliorates skin edema of acute skin inflammation model induced by 12-O-tetradecanoylphorbol-13-acetate (TPA) in mice (Kadoshima-Yamaoka et al., 2009a).

In the present study, we provide the data suggesting that ASB16165 inhibits not only the increase of the skin and epidermal thickness but also decreases the number of Ki67-positive keratinocytes in a mouse chronic skin inflammation model, which is induced by multiple topical application of TPA. Our results also demonstrated that ASB16165 suppresses proliferation of human keratinocytes in vitro. These findings strongly suggest that PDE7A is a valuable therapeutic target of the skin diseases, in which keratinocytes have a pathogenic role, *e.g.* psoriasis.

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2. Materials and methods

2.1. Mice

Female BALB/cAnNCrlCrlj (BALB/c) mice were obtained from Charles River Laboratories Japan, Inc. (Tokyo, Japan). All mice were housed in a specific pathogen-free environment and used between 7 and 9 weeks of age. All animal experiments were approved by the Ethics Committee for Animal Experiments of Biomedical Research Laboratories, Asubio Pharma Co., Ltd. and were performed in accordance with the Guideline for Animal Experiments of the laboratories.

2.2. Materials

ASB16165 (1-Cyclohexyl-*N*-[6-(4-hydroxy-1-piperidinyl)-3-pyridinyl]-3-methyl-1H-thieno[2,3-*c*]pyrazole-5-carboxamide monohydrate), which showed potent inhibitory activity to recombinant PDE7A with an IC50 value of 15 nM, was synthesized at Asubio Pharma Co., Ltd. (Osaka, Japan). The effect of ASB16165 was specific in that the effects on the other PDEs were little or only marginal; the IC50 for PDE4 was 2.1 μM (approximately 140-fold higher than that for PDE7A), and IC50s for the other PDEs (PDE2A3, PDE3, PDE5, PDE8A1, PDE9A2 and PDE10A1) were higher than 10 μM. TPA and betamethasone dipropionate were purchased from Wako Pure Chemical Industries Ltd. (Osaka, Japan). Human epidermal keratinocytes were purchased from Lonza Walkersville (Walkersville, MD) and cultured in KBM-2 medium supplemented with bovine pituitary extract (BPE), human epidermal growth factor (hEGF), insulin, and antibiotic GA-1000. Rolipram, dibutyryl cAMP (db-cAMP) and 1α,25-Dihydroxyvitamine D3 (vitamin D3) were purchased from Sigma-Aldrich Japan K.K (Tokyo, Japan). Bromodeoxyuridine (BrdU) cell proliferation ELISA was obtained from Roche Diagnostics (Mannheim, Germany).

2.3. Mouse skin inflammation induced by multiple topical applications of TPA

Effects of test compounds on TPA-induced skin inflammation were examined as described previously (Delescluse et al., 1987) with a slight modification. Briefly, 20 μl of TPA solution (10 μg/ml of TPA in 0.2% dimethylsulfoxide/99.8% methanol) was applied topically on the right ear of BALB/c mice on days 0, 2, 4, 7 and 9, and ear thickness was measured every day except for days 5 and 6 using a thickness gauge (Digimatic Indicator, Mitutoyo, Tokyo, Japan). The increase in ear thickness was determined by subtracting the ear thickness before painting from that of each time point. In the control group, solvent (0.2% dimethylsulfoxide/99.8% methanol) was applied instead of TPA solution. To examine the effects of ASB16165 or betamethasone, each compound was dissolved in the TPA solution, and the solution was painted onto the ears instead of the TPA solution. On the days except those of the TPA application, each compound was dissolved in methanol and applied.

2.4. Histological analysis

Ear samples excised on day 10 of the TPA-induced skin inflammation model were fixed in 10% (v/v) buffered formalin, embedded in paraffin, and sectioned at 4 μm. The sections were then stained with hematoxylin and eosin. The thickness of the epidermis (from the basal layer to stratum corneum) was measured at 30 random sites along the length of the specimen (approximately 1 mm) using Lumina Vision version 2. 5. 0.15 (Mitani Corp., Tokyo, Japan).

2.5. Immunostaining

Immunohistochemical staining was performed on paraformaldehyde-fixed, paraffin-embedded ear sections which were prepared with the ear samples excised on day 10 of the TPA-induced skin inflammation model. The sections were initially deparaffinized with xylene and rehydrated through graded alcohols. Antigen-unmasking in the sections was then performed by pretreatment with 0.05% Tween 20 for 5 min. After quenching the endogenous peroxidase activity by incubation in 3% H_2O_2 for 10 min, the sections were incubated overnight with anti-mouse Ki67 antibody (M7249, Dako Japan, Tokyo) at 4 °C. After washing, the sections were incubated with Simple Stain Mouse MAX-PO (414311, Nichirei, Corp., Tokyo, Japan) at room temperature for 30 min. To visualize the immune complex, 3,3′-diaminobenzidine (K3468, Dako Japan) was used, and the sections were counterstained with hematoxylin. The Ki67 positive cells in the epidermis were counted along the approximately 1 mm length of the specimen.

2.6. Cell proliferation of human keratinocytes in vitro

Human epidermal keratinocytes (2×10^3 cells/well) were cultured in 96 well collagen I-coated flat bottom plate for 18 h before the experiments. After removing the supernatant and adding the fresh medium, each test compound was added to the culture. Following an additional 48 h culture, BrdU incorporation assay was performed using colorimetric BrdU cell proliferation kit according to the manufacture's protocol.

2.7. Statistical analysis

The data were presented as mean ± S.D. Statistical analysis was performed by Student's *t*-test or Dunnett's multiple comparison test by using EXSUS version 7.5.2 (Arm Systex Co., Ltd, Osaka, Japan). The *P* value less than 0.05 was considered to be significant.

3. Results

3.1. Effect of ASB16165 on increase of ear thickness induced by multiple topical application of TPA

First, the effect of ASB16165 on skin inflammation was examined using a skin inflammation model induced by repeated topical application of TPA, which is characterized by prolonged skin reaction and epidermal hyperplasia. As shown in Fig. 1, the topical application of TPA to mouse ear resulted in increase of the ear thickness. ASB16165 reduced the TPA-induced increase of ear thickness, *e.g.* 1.0%

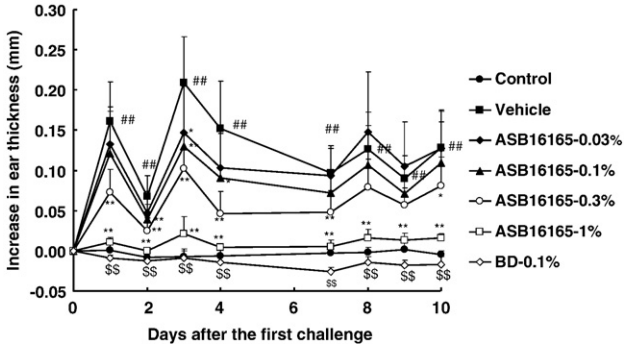

Fig. 1. Effect of ASB16165 on increase of ear thickness induced by multiple topical application of TPA. 20 μl of TPA (10 μg/ml) was applied topically to both sides of the right ear of BALB/c mice on days 0, 2, 4, 7 and 9. ASB16165 or betamethasone (BD) was topically applied to the right ears everyday in the same way. Ear thickness was measured using a thickness gauge, and the increase in ear thickness was determined as described in the Materials and methods. The data are expressed as mean ± S.D. ($N=10$). Control, solvent for TPA was applied instead of TPA solution. Vehicle, TPA but not any compound was applied. ## $P<0.01$, compared with control (Student's *t*-test). * $P<0.05$, ** $P<0.01$, compared with vehicle (Dunnett's test). $$ $P<0.01$, compared with vehicle (Student's *t*-test).

of ASB16165 showed almost complete inhibition with statistical significance at all the measure points, whereas the inhibitory effect was significant at 0.03% and higher doses on day 3 (Fig. 1). The ears of the mice treated with 0.1% of betamethasone became thinner along with the treatment, even as compared with those before the treatment as well as those treated with the solvent alone. It should be noted that body weight was remarkably reduced by the treatment with betamethasone but not with ASB16165; the averages of body weights of the mice treated with vehicle, 1.0% ASB16165 and 0.1% of betamethasone were 101.5%, 101.2% and 89.4%, respectively, at the end of the experiments, as compared with those at the start of the experiment.

3.2. Effect of ASB16165 on increase of epidermal thickness induced by multiple topical application of TPA

Histological analysis performed on day 10 demonstrates that multiple topical application of TPA increases the epidermal thickness (Fig. 2a), consistent with the previous report (Delescluse et al., 1987). The observation suggests that excessive proliferation of epidermal keratinocytes, in addition to dermal hyperplasia, contributes to the increase of ear thickness (Fig. 1). Measurement of the thickness of the epidermis from the basal layer to the stratum corneum revealed that the epidermal thickness in TPA-treated mice is approximately 2.4-fold that of the control mice (Fig. 2b). ASB16165 reduced the increase of epidermal thickness, and statistical significance was detected at the doses at 0.3% and higher (Fig. 2b). Betamethasone also exhibited a potent and significant inhibitory effect on the increase of the epidermal thickness.

3.3. Effect of ASB16165 on proliferation of epidermal keratinocytes in vivo

To determine the effect of ASB16165 on cell proliferation in the TPA model, the ear sections were stained for Ki67, the nuclear proliferation antigen. As shown in Fig. 3, the multiple topical application of TPA increased the Ki67-positive cells on day 10. Most of the positive cells were detected in the basal layer of the epidermis (Fig. 3a), suggesting the promotion of keratinocyte proliferation by

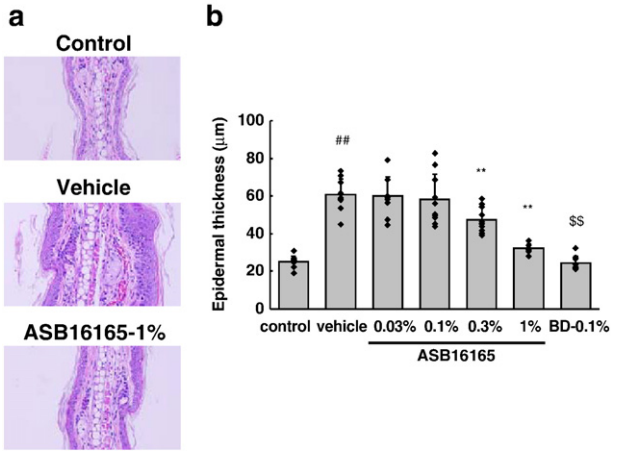

Fig. 2. Effect of ASB16165 on increase of epidermal thickness induced by multiple topical application of TPA. 20 μl of TPA (10 μg/ml) was applied topically to both sides of the right ear of BALB/c mice on days 0, 2, 4, 7 and 9. ASB16165 or betamethasone (BD) was topically applied to the right ears everyday in the same way. Ear samples were excised on day 10 and subjected to histological analysis. Epidermal thickness was measured as described in the Materials and methods. (a) Representative vertical sections stained with hematoxylin and eosin (×200). (b) Quantitative analysis of the effect of ASB16165 on the increase of epidermal thickness. The data are expressed as mean ± S.D. ($N = 10$) and square symbols represent each value of 10 mice. Control, solvent for TPA was applied instead of TPA solution. Vehicle, TPA but not any compound was applied. ## $P<0.01$, compared with control (Student's t-test). ** $P<0.01$, compared with vehicle (Dunnett's test). $$ $P<0.01$, compared with vehicle (Student's t-test).

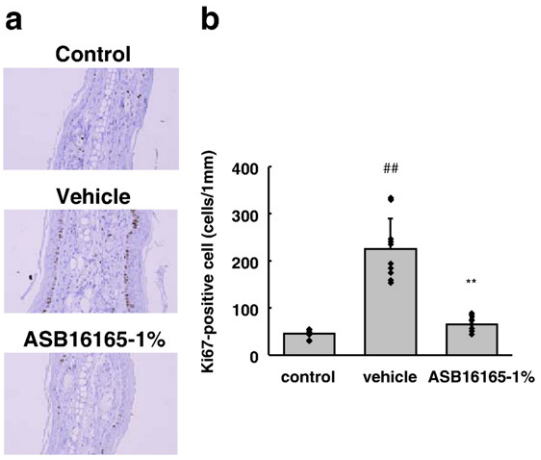

Fig. 3. Effect of ASB16165 on increase of Ki67 positive cells in epidermis induced by multiple topical application of TPA. 20 μl of TPA (10 μg/ml) was applied topically to both sides of the right ear of BALB/c mice on days 0, 2, 4, 7 and 9. ASB16165 or betamethasone was topically applied to the right ears everyday in the same way. Ear samples were excised on day 10 and subjected to the immunostaining for Ki67. The Ki67 positive cells in the epidermis were counted as described in the Materials and methods. (a) Representative photo of ear sections stained with anti-Ki67 antibody (×200). (b) Quantitative analysis of the effect of ASB16165 on the number of Ki67 positive cells in epidermis. The data are expressed as mean ± S.D. ($N = 10$) and square symbols represent each value of 10 mice. Control, solvent for TPA was applied instead of TPA solution. Vehicle, TPA but not any compound was applied. ## $P<0.01$, compared with control (Student's t-test). ** $P<0.01$, compared with vehicle (Student's t-test).

TPA. This is also in agreement with the fact that the dividing keratinocytes are located at the stratum basale, while the cells that start differentiation migrate towards the surface layers in the epidermis (Eckert, 1989). The treatment of ASB16165 dramatically decreased the Ki67-positive cells in epidermis, and counting the number of the Ki67-positive cells showed that the number of Ki67-positive cells in the ASB16165-treated mice was approximately 29% of that of the vehicle group (Fig. 3b).

3.4. Effect of ASB16165 on proliferation of human epidermal keratinocytes in vitro

As shown in Fig. 4, ASB16165 inhibited the proliferation of human keratinocytes in vitro in a concentration-dependent manner, and the IC50 value was approximately 1.8 μM. Vitamin D3 also showed the inhibitory effect, while betamethasone did not. This result indicates that the effect of betamethasone in the TPA-induced chronic dermatitis model is mediated by the mechanism(s) other than the

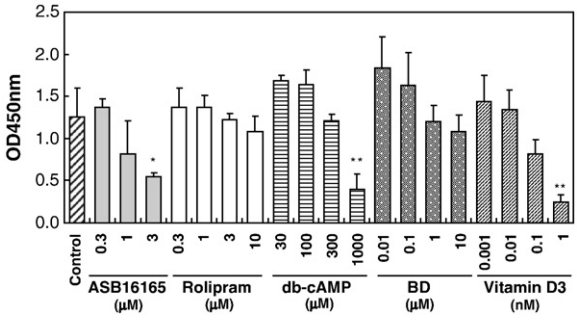

Fig. 4. Effect of ASB16165 on cell proliferation of human epidermal keratinocytes in vitro. Human keratinocytes were incubated with various concentrations of ASB16165, rolipram, db-cAMP, betamethasone (BD) and vitamin D3 in 96-well collagen I-coated flat bottom plate. After incubation for 48 h, cell proliferation was analyzed with BrdU incorporation assay. The data are expressed as mean ± S.D. of triplicate cultures. ** $P<0.01$, * $P<0.05$, compared with the control group in which no test compound was added (Dunnett's test).

inhibition of keratinocyte proliferation. Dibutyryl cAMP (db-cAMP) also showed the inhibition in the assay, suggesting the involvement of cAMP in the proliferation of human keratinocytes. A PDE4-specific inhibitor rolipram failed to suppress the proliferation of the cells. These findings indicate that PDE7A might be associated with the proliferation of keratinocyte probably by regulating the level of cellular cAMP.

4. Discussion

It is well known that cAMP inhibits proliferation of a number of cell types (Frodin et al., 1994; Sevetson et al., 1993; Withers et al., 1995), but rather stimulates proliferation of the others (Burgering et al., 1993; Cook and McCormick, 1993). The effects of cAMP on cell proliferation is largely due to how cAMP interacts with the RAS/RAF/MEK/ERK signaling pathways which regulate cell proliferation in response to various stimuli including growth factors (Dumaz and Marais, 2005; Stork and Schmitt, 2002). Namely, inhibition of C-RAF by cAMP results in prevention of the ERK activity, whereas cAMP activates B-RAF, leading to the stimulation of ERK. The effect of cAMP might depend on which RAF isozyme exists near the site of cAMP accumulation, if cAMP is produced in a highly localized manner, and if stimulation of RAS/RAF/MEK/ERK pathway by growth factor(s) concurs with cellular cAMP elevation.

In keratinocytes, the effect of cAMP on their proliferation in culture has been reported to be associated with the cell density, *i.e.* cAMP increases keratinocyte proliferation in the logarithmic growth phase, but decreases proliferation under confluent culture condition (Okada et al., 1982). In the present study, however, PDE7A inhibitor ASB16165 as well as db-cAMP significantly inhibited the proliferation of human keratinocytes in vitro in subconfluent condition (Fig. 4). Thus, the effects of cAMP on keratinocyte proliferation in vitro may also be influenced by the other factor(s) such as the concentrations of some growth factors in the culture medium, considering the importance of the crosstalk between cAMP and RAS/RAF/MEK/ERK pathways in proliferation as described above. In this respect, our data suggest that elevation of cAMP by PDE7A inhibitor might occur close to the site where C-RAF localizes. It is also important to mention that forskolin did not affect the keratinocyte proliferation (data not shown), showing that forskolin might increase the cAMP at the site apart from C-RAF.

In this study, multiple painting of TPA to the mouse skin stimulated keratinocyte proliferation, which was inhibited significantly by the ASB16165 treatment (Fig. 3). This result coincides with the effect of the compound on human keratinocytes in vitro, and indicates that the culture condition used in this study reflects the pathophysiological environment of the skin after the repeated topical application with TPA. The result also shows that PDE7A inhibitors could show antiproliferative effect on keratinocytes in the skin lesion of psoriasis patients, since the TPA model has been used as an animal model of the disease (Gupta et al., 1988).

In our study, on the other hand, the possibility that the mechanism(s) other than cAMP pathway participates in the effect of PDE7A inhibitor ASB16165 on keratinocyte proliferation has not been excluded completely, since a protein kinase A inhibitor (Rp-8-bromo-cAMP-phosphothioate) as well as adenylyl cyclase inhibitors (SQ22536, 2′,5′-dideoxyadenosine) failed to reverse the effect of ASB16165 on proliferation of human keratinocytes even at 1.0 mM (data not shown). Interestingly, in fact, rolipram (a PDE4 inhibitor), which elevates cAMP in keratinocytes (Wettey et al., 2006), did not show the inhibitory effect (Fig. 4). Moreover, the IC50 value of ASB16165 for keratinocyte proliferation (1.8 μM) is much higher than the values for PDE7A (1.5 nM) and cytokine production in lymphocytes (Goto et al., 2009). Further studies are needed to elucidate whether cAMP plays an essential role in the effect of ASB16165 on keratinocyte proliferation.

An alternative or additional mechanism(s) underlying the antiproliferative effect of ASB16165 on keratinocytes might be an indirect effect mediated by inhibition of cytokine production, while cAMP pathway would probably contribute to the mechanism as well. For instance, proliferation of keratinocytes is also known to be controlled in autocrine and paracrine fashions; nerve growth factor (NGF), which promotes keratinocyte proliferation, is produced by keratinocytes, and anti-NGF antibody reduces the proliferation of keratinocytes in culture where NGF is not added, suggesting an NGF-mediated autocrine loop contributing to keratinocyte proliferation (Dumaz and Marais, 2005). Keratinocyte-derived IL-1α and -1β stimulate release of keratinocyte growth factor (KGF) and GM-CSF from fibroblasts, and KGF and GM-CSF in turn stimulate keratinocyte proliferation (Werner and Smola, 2001). Furthermore, our recent studies have shown that PDE7A inhibitor ASB16165 suppresses production of several cytokines in various cell types including keratinocytes (Goto et al., 2009; Kadoshima-Yamaoka et al., 2009a).

In summary, the data in the present study suggested that PDE7A is related to the proliferation of epidermal keratinocytes presumably through controlling the cellular cAMP level. The PDE7A inhibitors such as ASB16165 could be useful as a novel agent for the treatment of the disease in which excessive growth of keratinocytes has a pathogenic role, like psoriasis.

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