Phosphatidic Acid Has a Potential to Promote Hair Growth *In Vitro* and *In Vivo*, and Activates Mitogen-Activated Protein Kinase/Extracellular Signal-Regulated Kinase Kinase in Hair Epithelial Cells

Tomoya Takahashi, Ayako Kamimura, Takako Hamazono-Matsuoka, and Shinkichi Honda*
Tsukuba Research Laboratories, Kyowa Hakko Kogyo Company, Tsukuba, Ibaraki, Japan, and *Bio-chemicals Company, Kyowa Hakko Kogyo Company, Ohtemachi, Tokyo, Japan

Phospholipids have recently been discovered to play an important role in cellular regulation. In this study, we focused on phosphatidic acid and lysophosphatidic acid, which are phospholipids known to possess growth-hormonal effects on several types of cells, and examined their growth-promoting effects on murine hair epithelial cells. We discovered that phosphatidic acid possesses intensive growth-promotional effects on hair epithelial cells and epidermal keratinocytes. In contrast, lysophosphatidic acid showed lower growth-promoting effects on hair epithelial cells relative to phosphatidic acid and showed minimal or no growth-promoting activity on epidermal keratinocytes. Phosphatidic acid was also shown to have hair-growing activity to induce the anagen phase of the hair cycle in the *in vivo* murine model. For the purpose of examining the hair-growing mechanisms of phosphatidic acid, we examined its relationship to the mitogen-activated protein kinase cascade linked to cell proliferation and the transforming growth factor β signal pathway known to be a regulator of catagen induction. We confirmed that phosphatidic acid activates MEK-1/2 and upregulates the expression of MEK-1/2 in cultured murine hair epithelial cells. Addition of transforming growth factor β1 to hair epithelial cell cultures concentration-dependently decreased cell growth and induced apoptosis; however, addition of phosphatidic acid to the culture neutralized the growth-inhibiting effects of transforming growth factor β1 and protected the cells from apoptosis. We speculate that the hair-growing activity of phosphatidic acid is at least linked to its growth-promoting effects on hair epithelial cells that follow mitogen-activated protein kinase/extracellular signal-regulated kinase kinase activation and its protective action on transforming-growth-factor-β1-induced apoptosis that is assumed to trigger catagen induction in the hair cycle. *Key words: apoptosis/MEK/phospholipids/transforming growth factor β. J Invest Dermatol 121:448–456, 2003*

Numerous researchers have investigated scientifically proven hair growth stimulants with respect to the mechanisms of action of how they exert hair-growing activity. One of these compounds, minoxidil (Rogaine®, Upjohn, Kalamazoo, MI), originally synthesized as a potassium channel opener (Buhl *et al*, 1993) and initially prescribed for hypertension, is an FDA-approved topical medication for treating male pattern baldness (Kulick, 1988; Olsen, 1989). Minoxidil is known to possess growth-promoting effects on hair epithelial cells (Tanigaki-Obana and Ito, 1992) and stimulates anagen induction of the hair cycle in animal models (Uno *et al*, 1985). The mechanisms of action by which minoxidil stimulates hair growth have not been fully elucidated; growth-factor-related mechanisms (Sanders *et al*, 1996; Lachgar *et al*, 1998; Yamazaki *et al*, 1999) and the effects of increased blood circulation in the capillary vessels (Hirkaler and Rosenberger, 1989) have been proposed. Cyclosporin A is a potent immunosuppressant, known to possess hair-growing activity by stimulating anagen induction in animal models (Paus *et al*, 1989), and which causes hirsutism in humans (Wysocki and Daley, 1987); it has been shown to possess growth-promoting effects on hair epithelial cells *in vitro* (Takahashi and Kamimura, 2001). We have recently found hair epithelial cell growth-promoting activity in proanthocyanidins such as procyanidin B-2, procyanidin B-3, and procyanidin C-1 (Takahashi *et al*, 1999; Kamimura and Takahashi, 2002) and have illustrated that these compounds have the potential to induce the anagen phase of the hair cycle in the *in vivo* murine model. In androgenetic alopecia, drugs targeting steroid receptors, steroid-metabolizing enzymes, growth factors, and cytokines have been investigated as potential agents for curing or treating this condition; hair follicular activation, such as hair epithelial cell growth promotion, may be an important index for evaluating the efficacy of hair growth stimulants (Shapiro and Price, 1998).

Concerns about biologically active phospholipids are attracting the interest of a growing number of researchers. Lysophosphatidic acid and phosphatidic acid are of particular interest. These two substances have been shown to exert growth-hormonal effects on numerous types of cells and to produce a range of cellular

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Reprint requests to: Tomoya Takahashi, Tsukuba Research Laboratories, Kyowa Hakko Kogyo Co., 2, Miyukigaoka, Tsukuba, Ibaraki 305-0841, Japan; Email: tomoya-goma@mx6.ttcn.ne.jp

responses in which receptor and nonreceptor-mediated mechanisms have been implicated (Nietgen and Durieux, 1998; Steed and Chow, 2001). Phosphatidic acid also has been identified as a second messenger (Liscovitch and Cantley, 1994; McPhail *et al*, 1999).

Phosphatidic acid is reported to promote growth of many types of cells, including mouse-embryo-derived fibroblast-like $C_3H/10T_{1/2}$ cells (Krabak and Hui, 1991), mouse-embryo-derived fibroblast-like Swiss 3T3 cells (Wood *et al*, 1993), rat fibroblast cell line Rat-1, human fibroblasts (van Corven *et al*, 1992), canine-kidney-derived epithelial-like MDCK cells (Bashir *et al*, 1992), human epidermoid carcinoma A431 cells (Kaszkin *et al*, 1992), endothelial cells (English *et al*, 2001), mesangial cells (Kester *et al*, 1989; Kester, 1993), cortical astrocyte cells (Pearce *et al*, 1994), and osteoblastic cells (Carpio and Dziak, 1998). Phosphatidic acid has also been shown to play an important role in controlling cell division through being involved in the mechanisms of G_1 to S transition (Flores *et al*, 1999).

On the other hand, lysophosphatidic acid has also been shown to promote the growth of many types of cells (Moolenaar, 1995): e.g., it has been reported to have growth-promoting activity on endothelial cells (Panetti *et al*, 1997; Lee *et al*, 2000a), smooth muscle cells (Cerutis *et al*, 1997), B lymphoblasts (Rosskopf *et al*, 1998), and fibroblasts (van Corven *et al*, 1989; 1992).

This paper describes our investigation of the effects of phosphatidic acid and lysophosphatidic acid on murine hair epithelial cells and epidermal keratinocytes. We examined the efficacy of these agents with respect to murine hair growth; and, for the first time, found that phosphatidic acid intensively promotes hair epithelial cell growth and stimulates anagen induction in the hair cycle progression *in vivo*. We also examined the mechanisms of action of the hair-growing activity stimulated by phosphatidic acid, focusing on its relation to activation of the mitogen-activated protein kinase cascade and transforming growth factor β1 (TGF-β1) induced cellular responses in hair epithelial cells, i.e., apoptotic cell death (Schuster and Krieglstein, 2002).

We speculate that the hair-growing activity of phosphatidic acid is at least linked to its growth-promoting effects on hair epithelial cells sequential to mitogen-activated protein kinase/extracellular signal-regulated kinase kinase (MEK) activation and its protective action on TGF-β1-induced apoptotic cell death that is assumed to trigger catagen induction (Seiberg *et al*, 1995; Foitzik *et al*, 2000) in the hair cycle.

MATERIALS AND METHODS

Materials Minoxidil, L-α-phosphatidyl-L-serine dipalmitoyl, L-α-phosphatidic acid (sodium salt, prepared from egg yolk lecithin), L-α-lysophosphatidylcholine (prepared from egg yolk lecithin), L-α-phosphatidylinositol (sodium salt, purified from soybeans), and L-α-phosphatidic acid dioleoyl (sodium salt) were purchased from Sigma Chemical (St Louis, MO). L-α-phosphatidylcholine dipalmitoyl, L-α-phosphatidylethanolamine dioleoyl, L-α-phosphatidylethanolamine dipalmitoyl, and L-α-phosphatidyl-DL-glycerol dipalmitoyl were purchased from Wako Pure Chemicals Industries (Osaka, Japan). L-α-lysophosphatidic acid oleoyl (sodium salt), L-α-phosphatidyl-L-serine dilauroyl (sodium salt), L-α-phosphatidic acid dilauroyl (sodium salt), L-α-phosphatidic acid dimyristoyl (sodium salt), L-α-lysophosphatidic acid myristoyl (sodium salt), and L-α-lysophosphatidic acid oleoyl (sodium salt) were purchased from Funakoshi (Tokyo, Japan). L-α-lysophosphatidic acid lauroyl (sodium salt) was prepared from L-α-phosphatidic acid dilauroyl (sodium salt, Funakoshi) using phospholipase A_2 by Tsuru Lecithin (Mie, Japan). Affinity-purified polyclonal antibodies against p-MEK-1/2 (goat antihuman) and against MEK-1/2 (rabbit antihuman) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). The secondary antibodies used were horseradish-peroxidase-conjugated rabbit antigoat immunoglobulin and horseradish-peroxidase-conjugated goat antirabbit immunoglobulin purchased from DAKO (Glostrup, Denmark).

Isolation and culturing of hair epithelial cells Murine hair epithelial cells were isolated from neonatal C3H/HeNCrj mice (Charles River Japan, Kanagawa, Japan) according to the method described in another report (Takahashi, 2001). Before starting our experiments, we confirmed, from the keratin pattern expressed in the cultured cells[1] that isolated cells were hair epithelial cells. The dorsal skin was peeled from 4-d-old C3H/HeNCrj mice, cut into about 5 mm widths, and then dipped into Eagle's minimum essential medium containing 750 IU per ml of dispase (from *Bacillus polymyxa*, Godo Shusei, Tokyo, Japan), 60 mg per L of kanamycin, and 10% (vol/vol) fetal bovine serum (FBS) at 4°C for 20 h. The epidermis was peeled off, and the remaining dermis layer was dispersed in Dulbecco's modified Eagle's medium (DMEM) containing 0.25% (wt/vol) collagenase (from *Streptomyces parvulus*, Nitta Gelatin, Osaka, Japan), 50,000 U per L of penicillin, 50 mg per L of streptomycin, 0.5% (wt/vol) bovine serum albumin, and 20% (vol/vol) FBS at 37°C for 1 h, stirring occasionally. This dermis suspension was filtered through a 212 μm nylon mesh, and the filtrate was centrifuged at 1400 rpm (400g) for 7 min. The pellet was resuspended in Dulbecco's phosphate-buffered calcium- and magnesium-free saline containing 50,000 U per L of penicillin and 50 mg per L of streptomycin (PBS-PS). The suspension was left to stand for 15 min, allowing the hair follicle tissue to precipitate, after which the supernatant was removed using an aspirator. The hair follicle tissue was resuspended in PBS-PS and then precipitated. This precipitation process was repeated three times. Finally, the hair follicle tissue was incubated in 0.05% (wt/vol) ethylenediamine tetraacetic acid (EDTA)−0.25% (wt/vol) trypsin in Hanks' balanced calcium- and magnesium-free salt solution (HBSS) (Life Technologies, MD) at 37°C for 5 min. The hair follicle cells were suspended in DMEM supplemented with 50,000 U per L of penicillin, 50 mg per L of streptomycin, and 10% (vol/vol) FBS at a density of 3×10^5 cells per ml after filtration via a 212 μm nylon mesh. This hair follicle cell suspension was pipetted into a 24-well type I collagen-coated plate (2 cm^2 per well, Iwaki Glass, Chiba, Japan) at a rate of 1 ml per well and incubated in a humidified atmosphere containing 5% CO_2 at 37°C for 24 h. After 24 h incubation, the medium was exchanged with MCDB 153 (Sigma) containing 5 mg per L of bovine insulin, 5 μg per L of mouse epidermal growth factor, 40 mg per L of bovine pituitary extract, 10 mg per L of human transferrin, 0.4 mg per L of hydrocortisone, 0.63 μg per L of progesterone, 14 mg per L of O-phosphorylethanolamine, 6.1 mg per L of ethanolamine, 50,000 U per L of penicillin, and 50 mg per L of streptomycin. It was then further incubated in a humidified atmosphere containing 5% CO_2 at 37°C. During incubation, the medium was removed and replaced with fresh medium every other day. After 5 d, the degree of cell growth was determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. For the control, we used a medium to which an amount of 1/100 volume of purified water or solvent was added in place of the phospholipid-containing solution. Growth-promoting activities relative to controls (=100%) were calculated. Results are represented as mean ± SD ($n = 6$) carried out with primary cultures.

Isolation and culturing of mouse epidermal keratinocytes The epidermis, obtained in the same manner as described for hair epithelial cells, was peeled off from the dispase-treated C3H/HeNCrj mouse skin, washed five times with PBS-PS, and then immersed in 0.05% (wt/vol) EDTA−0.25% (wt/vol) trypsin in HBSS at 37°C for 10 min, stirring occasionally. After 10 min, DMEM supplemented with 50,000 U per L of penicillin, 50 mg per L of streptomycin, and 10% (vol/vol) FBS was added, and the suspension was filtered through a 212 μm nylon mesh, followed by centrifugation at 1500 rpm for 5 min. The keratinocytes thus obtained were cultured in a 24-well type I collagen-coated plate (at an initial cell density of 1.5×10^5 cells per cm^2, Iwaki Glass) in the same manner as described for hair epithelial cells using DMEM for the first 24 h and next MCDB 153 (containing the same additives as for the hair epithelial cells) for 6 d.

Colorimetric assay for cell proliferation by MTT The degree of cell growth was determined by MTT assay (Carmichael *et al*, 1987). To summarize, MTT reagent was dissolved in Dulbecco's phosphate-buffered calcium- and magnesium-free saline (PBS) at a concentration of 5 mg per ml, filtered through a 0.45 μm membrane filter (cellulose acetate, DISMIC-13 cp, Advantec, Tokyo, Japan), and added 10% (vol/vol) to the culture medium. The culture plate was further incubated in a humidified atmosphere containing 5% CO_2 at 37°C for 4 h. After removing the medium, the formed dye was extracted with acidic isopropanol containing 0.04 N HCl (adding 1.0 ml per 2 cm^2 well), and the absorbance was measured at 570 nm relative to 640 nm. As controls, we

[1]Tanigaki N, Nakamura S, Kanehisa H, Masamoto Y, Kitano Y: Methods for optimized cultivation of hair cells from C3H mice. *Japanese Journal of Dermatology* 99:1145–1152, 1989 (Japanese Journal).

placed cell-free blanks, in which test-agent-containing medium was pipetted, in the culture plates.

Preparation of topically applied agents for *in vivo* evaluation Test samples were dissolved in basal solvent comprising 80% (wt/wt) ethyl alcohol, 5% (wt/wt) 1,3-butylene glycol, 0.5% (wt/wt) N-acetylglutamine isostearyl ester (Kyowa Hakko Kogyo, Tokyo, Japan), 0.25% (wt/wt) polyoxyethylene (25) glyceryl monopyroglutamate monoisostearate (Nihon Emulsion, Tokyo, Japan), 0.2% (wt/wt) dl-α-tocopherol (Roche, Basel, Switzerland), 0.02% (wt/wt) d-biotin (Roche), and pure water making up to 100%; they were subjected to *in vivo* mouse testing. Basal solvent (= vehicle) was used as the control.

Test for hair-growing activity by induction of the anagen phase using the C3H mouse model The degree of hair-growing activity by induction of the anagen phase was measured using C3H mice with reference to the method introduced by Hattori and Ogawa (1983). In this test, 8-wk-old male C3H/HeSlc mice (Japan SLC, Shizuoka, Japan) whose hair cycle was in the telogen phase were used. The hair on the back of each mouse was carefully shaved with an electric shaver so as not to injure or stimulate the skin. Two hundred microliters daily of test sample was applied to the shaved area. On the eighteenth day of the test, the mouse back skin was observed and photographed; then the skin was peeled from the back of each mouse and photographed.

Immunoblot analysis (western blotting) The cultured murine hair epithelial cell pellet was sonicated in five 10 s bursts in buffer A (20 mM tris(hydroxymethyl)aminomethane (Tris)−HCl (pH 7.5), 2 mM EDTA, 10 mM ethyleneglycol-bis(β-aminoethyl ether)-N,N,N′,N′-tetraacetic acid, 0.25 M sucrose, 2 mM phenylmethylsulfonyl fluoride, 10 μg per ml leupeptin, and 10 mM 2-mercaptoethanol; final concentrations) and centrifuged at $100{,}000 \times g$ for 60 min (4 °C). The supernatants were subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis and immunoblot analysis according to the method described in another report (Takahashi and Kamimura, 2001) with minor modifications. For the detection of p-MEK-1/2 and MEK-1/2, we used polyclonal antibody against p-MEK-1/2 (goat antihuman, Santa Cruz Biotechnology) and MEK-1/2 (rabbit antihuman, Santa Cruz Biotechnology) respectively as primary antibody, and we used horseradish-peroxidase-conjugated rabbit antigoat immunoglobulin (DAKO) and horseradish-peroxidase-conjugated goat antirabbit immunoglobulin (DAKO) respectively as secondary antibody.

Detection of apoptotic cell death in cultured hair epithelial cells using ELISA Apoptotic cell death was quantitatively measured by detecting histone-associated DNA fragments using the sandwich ELISA system (Cell Death Detection ELISA^{PLUS}, Boehringer Mannheim). Both the cell lysate and the culture medium supernatant were subjected to analysis.

RESULTS

L-α-phosphatidic acid showed intensive growth-promoting action on hair epithelial cells We examined the growth-promoting activity of several phospholipids on hair epithelial cells. We selected L-α-phosphatidic acid prepared from egg yolk lecithin, L-α-phosphatidylcholine dipalmitoyl, L-α-phosphatidylethanolamine dioleoyl, L-α-phosphatidylethanolamine dipalmitoyl, L-α-phosphatidyl-DL-glycerol dipalmitoyl, L-α-phosphatidylinositol purified from soybeans, and L-α-phosphatidyl-L-serine dilauroyl, L-α-phosphatidyl-L-serine dipalmitoyl, L-α-lysophosphatidic acid oleoyl, and L-α-lysophosphatidylcholine prepared from egg yolk lecithin as typical compounds. We performed the *in vitro* experiments over a wide range of concentrations (from 0.1 μM to 500 μM for synthetic products; 100 ng per ml to 100 μg per ml for natural products), which included the physiologic concentrations of these phospholipids in serum (Sasagawa *et al*, 1998). **Figure 1** shows the growth-promoting activity of these phospholipids on hair epithelial cells. Minoxidil showed maximal growth-promoting activity of about 160% at the optimum concentration of 400 μM (162% ± 2% (average ± SD); two-sample *t* test, $p < 0.001$). Of the phospholipids examined, L-α-phosphatidic acid prepared from egg yolk lecithin showed the highest growth-promoting activity of about 300% on hair epithelial cells (14 μg per ml, 287% ± 21% (average ± SD); two-sample *t* test, $p < 0.001$). L-α-phosphatidylcholine dipalmitoyl (50 μM, 157% ± 8% (average ± SD); two-sample *t* test, $p < 0.001$), L-α-phosphatidylethanolamine dioleoyl (5 μM, 142% ± 19% (average ± SD); two-sample *t* test, $p < 0.002$), L-α-phosphatidyl-L-serine dipalmitoyl (50 μM, 145% ± 27% (average ± SD); two-sample *t* test, $p < 0.005$), and L-α-lysophosphatidic acid oleoyl (1 μM, 135% ± 5% (average ± SD); two-sample *t* test, $p < 0.001$) showed relatively lower growth-promoting activity on hair epithelial cells of about 140% relative to the controls (= 100%). L-α-phosphatidylethanolamine dipalmitoyl, L-α-phosphatidyl-DL-glycerol dipalmitoyl, L-α-phosphatidylinositol purified from soybeans, L-α-phosphatidyl-L-serine dilauroyl, and L-α-lysophosphatidylcholine prepared from egg yolk lecithin showed no growth-promoting effects on hair epithelial cells. Among the examined phospholipids, phosphatidic acid was the only compound that showed a high level of growth-promoting activity on hair epithelial cells.

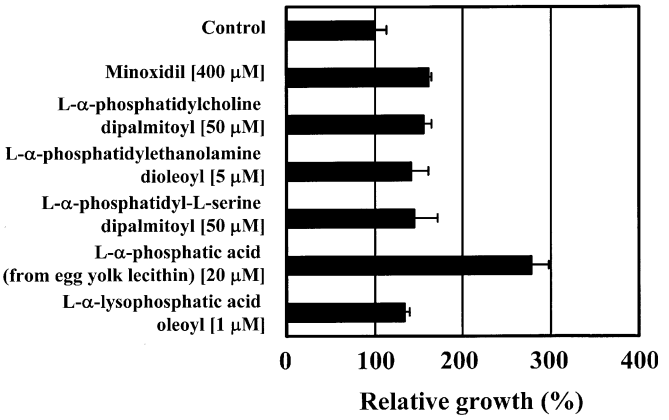

Figure 1. L-α-phosphatidic acid selectively and intensively promotes the growth of hair epithelial cells. We examined the growth-promoting activity on hair epithelial cells by several phospholipids. Maximum growth-promoting activities on hair epithelial cells relative to controls (= 100%) are shown. L-α-phosphatidyl-L-serine dilauroyl was dissolved in purified water and added at a rate of 1% (vol/vol) to the MCDB 153 medium. Minoxidil, L-α-phosphatidylethanolamine dioleoyl, and L-α-phosphatidyl-L-serine dipalmitoyl were dissolved in dimethylsulfoxide and added at a rate of 1% (vol/vol) to the MCDB 153 medium. L-α-phosphatidic acid prepared from egg yolk lecithin, L-α-lysophosphatidic acid oleoyl, and L-α-lysophosphatidylcholine prepared from egg yolk lecithin were dissolved in methanol and added at a rate of 1% (vol/vol) to the MCDB 153 medium. L-α-phosphatidylcholine dipalmitoyl, L-α-phosphatidylethanolamine dipalmitoyl, L-α-phosphatidyl-DL-glycerol dipalmitoyl, and L-α-phosphatidylinositol purified from soybeans were dissolved in ethanol and added at a rate of 1% (vol/vol) to the MCDB 153 medium. Brackets show optimum concentration. Results are represented as mean ± SD ($n = 6$) carried out with primary cultures prepared from 50 neonatal mice.

L-α-phosphatidic acids intensively promote hair epithelial cell growth; however, only weak growth-promoting activity was seen for L-α-lysophosphatidic acids We examined the growth-promoting activity on hair epithelial cells by phosphatidic acids and lysophosphatidic acids possessing different fatty acid residues: lauroyl, myristoyl, and oleoyl. L-α-phosphatidic acid dilauroyl (379% ± 26%, average ± SD), L-α-phosphatidic acid dimyristoyl (361% ± 17%, average ± SD), and L-α-phosphatidic acid dioleoyl (371% ± 66%, average ± SD) all showed intensive growth-promoting activity of about 350% relative to controls (= 100%) at a concentration of 10 μM (two-sample *t* test, $p < 0.001$). At concentrations lower than 10 μM, the growth-promoting activity of L-α-phosphatidic acid dioleoyl was

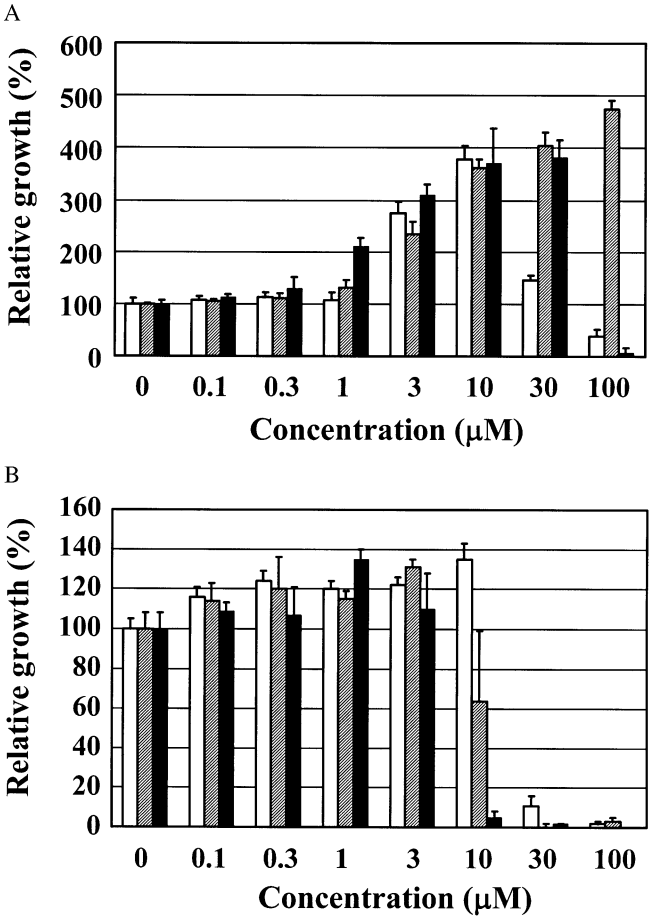

Figure 2. L-α-phosphatidic acid dilauroyl, L-α-phosphatidic acid dimyristoyl, and L-α-phosphatidic acid dioleoyl all intensively promote hair epithelial cell growth; L-α-lysophosphatidic acids slightly promote the growth of hair epithelial cells. We examined the growth-promoting activity on hair epithelial cells of phosphatidic acids (*A*) and lysophosphatidic acids (*B*) possessing different fatty acid residues. (*A*) L-α-phosphatidic acid dilauroyl and L-α-phosphatidic acid dimyristoyl were dissolved in methanol and added at a rate of 1% (vol/vol) to the MCDB 153 medium. L-α-phosphatidic acid dioleoyl was dissolved in water and added at a rate of 1% (vol/vol) to the MCDB 153 medium. Growth-promoting activities of L-α-phosphatidic acid dilauroyl (*clear bar*), L-α-phosphatidic acid dimyristoyl (*hatched bar*), and L-α-phosphatidic acid dioleoyl (*solid bar*) are shown. (*B*) L-α-lysophosphatidic acid lauroyl, L-α-lysophosphatidic acid myristoyl, and L-α-lysophosphatidic acid oleoyl were dissolved in water and added at a rate of 1% (vol/vol) to the MCDB 153 medium. Growth-promoting activities of L-α-lysophosphatidic acid lauroyl (*clear bar*), L-α-lysophosphatidic acid myristoyl (*hatched bar*), and L-α-lysophosphatidic acid oleoyl (*solid bar*) are shown.

higher than those of L-α-phosphatidic acid dilauroyl and L-α-phosphatidic acid dimyristoyl. At concentrations of 100 μM, the growth-promoting activity of L-α-phosphatidic acid dimyristoyl showed the highest growth-promoting activity of about 500% relative to the controls (=100%) (474%±17% (average±SD); two-sample *t* test, $p<0.001$) (**Fig 2*A***). On the other hand, L-α-lysophosphatidic acid lauroyl (10 μM, 135%±8% (average±SD); two-sample *t* test, $p<000.1$), L-α-lysophosphatidic acid myristoyl (3 μM, 131%±4% (average±SD); two-sample *t* test, $p<0.001$), and L-α-lysophosphatidic acid oleoyl (1 μM, 135%±5% (average±SD); two-sample *t* test, $p<0.001$) showed a maximal growth-promoting activity of about 130%, lower than those of phosphatidic acids (**Fig 2*B***).

L-α-phosphatidic acid dioleoyl intensively promotes the growth of epidermal keratinocytes; in contrast, all the L-α-lysophosphatidic acids tested showed minimal or no growth-promoting activity on epidermal keratinocytes We examined the effect of L-α-phosphatidic acid dilauroyl, L-α-phosphatidic acid dimyristoyl, L-α-phosphatidic acid dioleoyl, L-α-lysophosphatidic acid lauroyl, L-α-lysophosphatidic acid myristoyl, and L-α-lysophosphatidic acid oleoyl on the proliferation of epidermal keratinocytes.

Figure 3(*A*) shows the effects of phosphatidic acids on the growth of keratinocytes. The results show that L-α-phosphatidic acid dioleoyl promotes the growth of keratinocytes at the maximal rate of about 350% relative to controls (=100%) at the optimal concentration of 30 μM (353%±61% (average±SD);

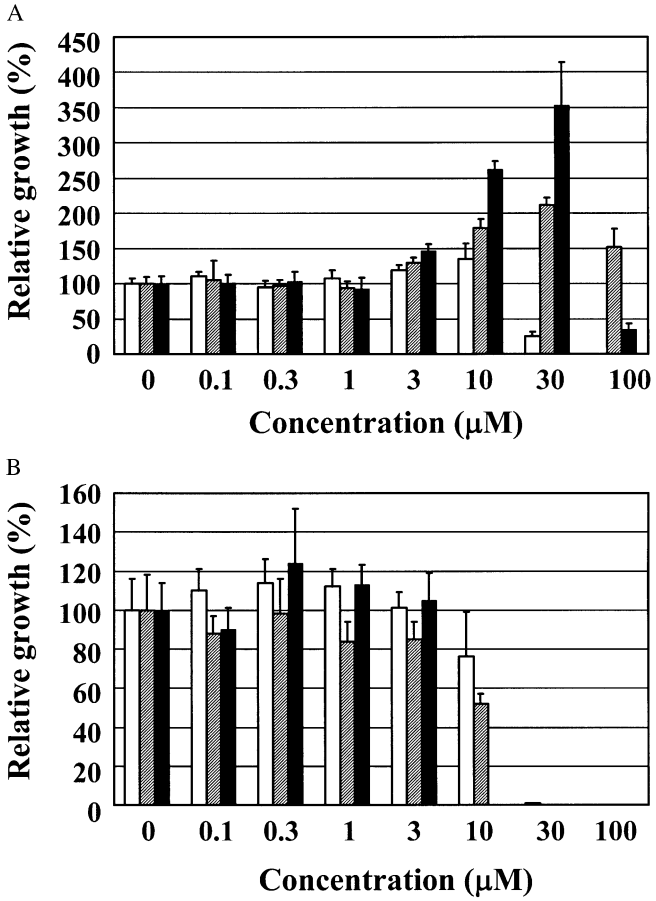

Figure 3. L-α-phosphatidic acid dioleoyl intensively promotes the growth of epidermal keratinocytes; in contrast, L-α-phosphatidic acid dilauroyl and L-α-phosphatidic acid dimyristoyl showed relatively lower activity; some L-α-lysophosphatidic acids slightly promote the growth of epidermal keratinocytes. We examined the growth-promoting activity on epidermal keratinocytes of phosphatidic acids (*A*) and lysophosphatidic acids (*B*) possessing different fatty acid residues. (*A*) L-α-phosphatidic acid dilauroyl and L-α-phosphatidic acid dimyristoyl were dissolved in methanol and added at a rate of 1% (vol/vol) to the MCDB 153 medium. L-α-phosphatidic acid dioleoyl was dissolved in water and added at a rate of 1% (vol/vol) to the MCDB 153 medium. Growth-promoting activities of L-α-phosphatidic acid dilauroyl (*clear bar*), L-α-phosphatidic acid dimyristoyl (*hatched bar*), and L-α-phosphatidic acid dioleoyl (*solid bar*) are shown. (*B*) L-α-lysophosphatidic acid lauroyl, L-α-lysophosphatidic acid myristoyl, and L-α-lysophosphatidic acid oleoyl were dissolved in water and added at a rate of 1% (vol/vol) to the MCDB 153 medium. Growth-promoting activities of L-α-lysophosphatidic acid lauroyl (*clear bar*), L-α-lysophosphatidic acid myristoyl (*hatched bar*), and L-α-lysophosphatidic acid oleoyl (*solid bar*) are shown.

two-sample *t* test, $p < 0.001$); in contrast, L-α-phosphatidic acid dilauroyl (10 μM, 135% ± 22% (average ± SD); two-sample *t* test, $p < 0.001$) and L-α-phosphatidic acid dimyristoyl (30 μM, 212% ± 10% (average ± SD); two-sample *t* test, $p < 0.001$) show relatively lower activity.

Figure 3(*B*) shows the effects of lysophosphatidic acids on the growth of keratinocytes. L-α-lysophosphatidic acid lauroyl (0.3 μM, 114% ± 12% (average ± SD); two-sample *t* test, $p < 0.1$) and L-α-lysophosphatidic acid oleoyl (0.3 μM, 124% ± 28% (average ± SD); two-sample *t* test, $p < 0.1$) are shown to slightly promote the growth of keratinocytes, about 120% at maximum; however, L-α-lysophosphatidic acid myristoyl inhibits the growth of keratinocytes at least at concentrations of above 1 μM.

L-α-phosphatidic acid stimulates anagen induction in hair cycle progression in the murine model C3H mouse dorsal hair is known to have a time-synchronized hair cycle. From about 18 to 21 d of age and 47 to 95 d of age, the dorsal hairs are in the telogen phase (Hattori and Ogawa, 1983). The test compound was topically applied from the eighth to the tenth week (18 d application) during the second telogen phase. The hair-covered area was evaluated at the tenth week.

In this assay system, minoxidil gave a positive response. The control group to which vehicle was applied showed little hair growth: only about 15% (17.1% ± 11.9%, average ± SD) of the shaved area was covered with hair on day 18. After an 18 d application of 1% (wt/wt) minoxidil-containing agent, however, about 75% (76.8% ± 20.5%, average ± SD) of the shaved area was covered with hair (two-sample *t* test, $p < 0.001$). The data are shown for the test of 0.4% (wt/wt) phospholipids applied, which is the maximum concentration of L-α-phosphatidic acid dioleoyl soluble in the preparation. The group to which 0.4% (wt/wt) L-α-phosphatidic acid dioleoyl-containing agent had been applied showed an extended growth area of 63.9% ± 19.9% (average ± SD) on day 18 (two-sample *t* test, $p < 0.001$). The group to which 0.4% (wt/wt) L-α-lysophosphatidic acid oleoyl-containing agent was applied, in contrast, showed little hair growth: only about 30% (28.9% ± 23.1%, average ± SD) of the shaved area was covered with hair on day 18 (**Fig 4**). These results demonstrate that phosphatidic acid possesses marked hair-growing activity to induce the anagen phase *in vivo*. In a histologic study, no adverse effects were observed in the epidermis, connective tissue, or hair follicles of C3H mouse back skin due to topical application of 0.4% (wt/wt) L-α-phosphatidic acid dioleoyl (**Fig 5**).

L-α-phosphatidic acid activates MEK-1/2 of cultured murine hair epithelial cells and upregulates the level of MEK-1/2 in cultured murine hair epithelial cells We examined the effects of phosphatidic acid on the level of p-MEK-1/2 and the expression of MEK-1/2 in cultured murine hair epithelial cells using western blotting. The hair epithelial cells were incubated in medium containing 10 μM of L-α-phosphatidic acid dioleoyl for the final 96 h of the 7 d culture period. The dose of L-α-phosphatidic acid dioleoyl for the investigation of p-MEK-1/2 and MEK-1/2 protein levels was set at 10 μM, at which hair epithelial cells show maximum growth-promoting activity.

We observed increases in the level of p-MEK-1/2 in hair epithelial cells cultured in medium containing 10 μM of L-α-phosphatidic acid dioleoyl by 318% ± 46% (average ± SD; paired *t* test, $p < 0.001$) relative to the controls (= 100%). The expression of MEK-1/2 in hair epithelial cells cultured in medium containing 10 μM of L-α-phosphatidic acid dioleoyl increased 1215% ± 392% (average ± SD; paired *t* test, $p < 0.001$) relative to the controls (= 100%) (**Fig 6**).

TGF-β1 concentration-dependently decreases hair epithelial cell growth and induces apoptosis in hair epithelial cells; addition of L-α-phosphatidic acid dioleoyl to the culture counteracts the inhibitory effect of TGF-β1 Of the many hair-growth-regulating factors known, TGF-β has of late been a focus of increasing research interest, as it is a potent negative hair-growing factor that induces the catagen phase of the hair cycle (Seiberg *et al*, 1995; Foitzik *et al*, 2000). We examined the effect of TGF-β1 on murine hair epithelial cell growth. We subsequently observed that addition of TGF-β1 to the culture medium concentration-dependently inhibits murine hair epithelial cell growth and induces apoptotic cell death. Addition of 10 μM of L-α-phosphatidic acid dioleoyl to medium containing 0.3 ng per ml of TGF-β1 completely counteracted the inhibitory effects of TGF-β1 and protected the cells from apoptosis (**Fig 7**). Micrographs of hair epithelial cells from mice cultured in the presence of TGF-β1 or L-α-phosphatidic acid dioleoyl or both are shown in **Fig 8**.

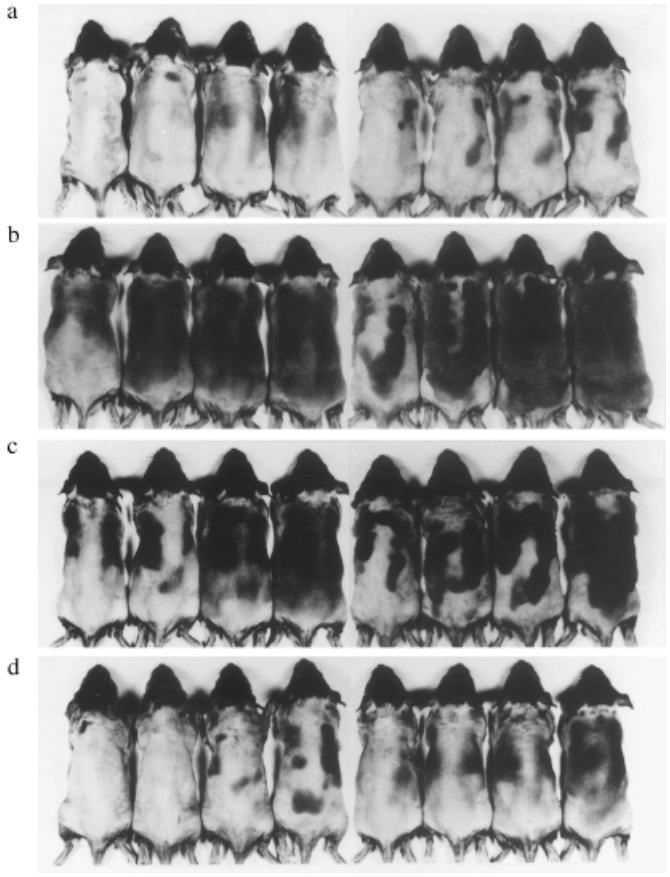

Figure 4. L-α-phosphatidic acid stimulates anagen induction in hair cycle progression in the murine model. Hair-growing activity that induced the anagen phase of the hair cycle was determined using the C3H mouse *in vivo* model. (*a*) Vehicle; (*b*) 1.0% (wt/wt) minoxidil; (*c*) 0.4% (wt/wt) L-α-phosphatidic acid dioleoyl; (*d*) 0.4% (wt/wt) L-α-lysophosphatidic acid oleoyl.

DISCUSSION

The hair follicle is composed of dermal papilla cells, which are classified as mesenchymal cells, and epithelial cells, such as the inner root sheath cells, outer root sheath cells, and hair matrix cells (Paus *et al*, 1999). The presence of hair follicular stem cells was assumed by Cotsarelis *et al* (1990) at the bulge area of the infundibular region of the outer root sheath. Mammalian hair is known to repeat what is known as the hair cycle: the growth stage (anagen) to resting stage (telogen) via a regression stage (catagen), then returning to the growth stage (anagen). Interactions between mesenchymal cells and epithelial cells are thought to be important in the progression of the hair cycle (Reynolds and

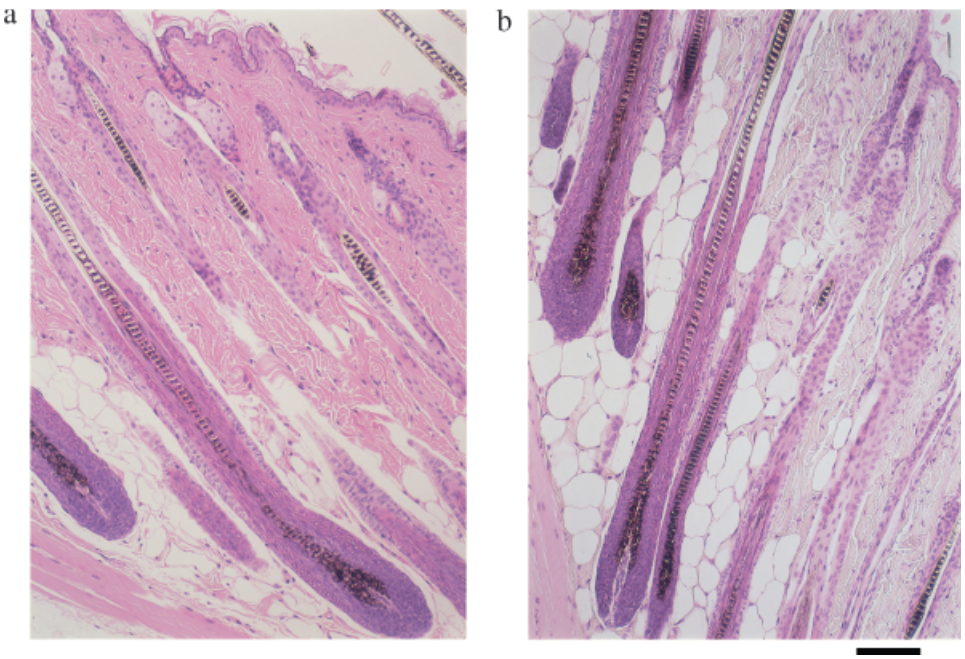

Figure 5. Hematoxylin and eosin stained sections of C3H mouse back skin after topical application of test agents for 18 d. Vehicle-treated skin section (*a*) of the hair-growing area and 0.4% (wt/wt) L-α-phosphatidic acid dioleoyl treated skin section (*b*) of the hair-growing area. *Scale bars*: 100 μm.

Jahoda, 1991a; Hardy, 1992). These mesenchymal–epithelial interactions are assumed to stimulate the germinative cells in hair follicles to proliferate and induce the anagen phase of the hair cycle (Tezuka *et al*, 1991); and these interactions are also assumed to activate stem cells by direct or indirect mechanisms and promote downgrowth of outer root sheath cells, resulting in the formation of mature hair follicles (Reynolds and Jahoda, 1991b).

In this paper, we have demonstrated that phosphatidic acid intensively promotes the proliferation of the hair follicular epithelium. On the other hand, we also demonstrated that phosphatidic acid promotes the proliferation of epidermal keratinocytes in skin. From this, we conclude that phosphatidic acid directly affects the growth of epithelial cells in skin. The chief hair-growing mechanism of action of phosphatidic acid is assumed to be a result of its intensive growth-promoting action on hair epithelial cells, presumably by inducing the anagen phase of the hair cycle by activation of the hair germ, promoting downgrowth of the hair follicles by activating the outer root sheath that includes the stem cells, and consequently leading to the formation of mature hair follicles.

Several internal factors are known to regulate hair growth (Danilenko *et al*, 1996): e.g., insulin-like growth factor 1, hepatocyte growth factor, keratinocyte growth factor, and vascular endothelial growth factor are known to positively regulate hair growth; TGF-β, fibroblast growth factor 5, tumor necrosis factor α, interleukin 1α, and interleukin 1β are known to negatively regulate hair growth.

Of these growth factors and cytokines reported to act on hair follicles, TGF-β1 is regarded as the likeliest candidate for regulating catagen induction of the hair cycle in the *in vivo* murine hair cycle (Seiberg *et al*, 1995; Foitzik *et al*, 2000). TGF-β1 is also known as an apoptosis-inducing factor (Schuster and Krieglstein, 2002) in many types of cells (Moustakas *et al*, 2002). As for its effects on skin keratinocytes, Shipley *et al* (1986) have reported that TGF-β causes G_1 arrest of the cell cycle in human keratinocytes; and Benassi *et al* (1997) also reported that TGF-β1 causes apoptosis in cultured human keratinocytes. We examined the effect of TGF-β1 on murine hair epithelial cell growth and observed that the addition of TGF-β1 to the culture medium concentration-dependently inhibits the growth of murine hair epithelial cells and induces apoptotic cell death. Addition of phosphatidic acid dioleoyl to medium containing TGF-β1 completely counteracted TGF-β1-induced apoptotic cell death (**Fig 7**). From these results, we conclude that the hair-growing mechanisms of phosphatidic acid are at least attributable to inhibition of TGF-β1-induced apoptotic cell death in hair epithelial cells.

Increasing interest is being shown by researchers in the biologically active functions of phospholipids, particularly phosphatidic acid and lysophosphatidic acid, which are suspected to play a potential role as intracellular second messengers and possess growth-hormonal effects on many types of cells and tissues. Research interest is growing in the signal transduction routes evoked by biologically active phospholipids. Raf-1 (Avruch *et al*, 1994; Daum *et al*, 1994; Force *et al*, 1994) has been identified (Ghosh *et al*, 1996; 1997; Ghosh and Bell, 1997) as a target of molecules in signal transduction pathways affected by phosphatidic acid that cause cell proliferation. Raf-1 is a ubiquitously expressed Ser/Thr kinase that plays an essential role in signal transduction from a large number of growth factor receptors, cytokine receptors, and G-protein-linked receptors (Morrison and Cutler, 1997). Phosphatidic acid binds to Raf-1 and enables it to attach to membranes, where it becomes active. Activation of Raf-1 results in the activation of a mitogen-activated protein kinase (MAPK) (= extracellular signal-regulated protein kinase (ERK)) cascade (Rizzo *et al*, 1999; 2000), an important pathway that occurs during cell proliferation. There are reports describing how phosphatidic acid activates Ras (Yu *et al*, 1988; Tsai *et al*, 1990), a proto-oncogene product, which interacts with Raf-1 and results in the activation of the MAPK cascade. Phosphatidic acid has also been shown to bind to G-protein coupled receptor (GPCR) to induce activation of the MAPK pathway (Alderton *et al*, 2001). One mechanism for the growth-promoting effect on hair epithelial cells of phosphatidic acid appears to be the activation of the MAPK signal route initiated by their interaction with their receptor (GPCR), G-protein (Ras), or its associated protein (Raf-1) on hair epithelial cells.

The mechanisms by which lysophosphatidic acid exerts its growth-hormonal effects are distinct from those of phosphatidic acid. Lysophosphatidic acid has been shown to bind to Edg receptors (Fukushima and Chun, 2001) and to activate Ras, Rho (Moolenaar *et al*, 1997), or Rac (Ueda *et al*, 2001) in each receptor-specific manner, and is believed to evoke a range of cellular responses, such as calcium mobilization, cellular proliferation, cellular contraction, tumor cell invasion, and platelet aggregation (Nietgen and Durieux, 1998).

In this report, we describe the inhibitory effect of phosphatidic acid on TGF-β-induced apoptosis in hair epithelial cells. Similar

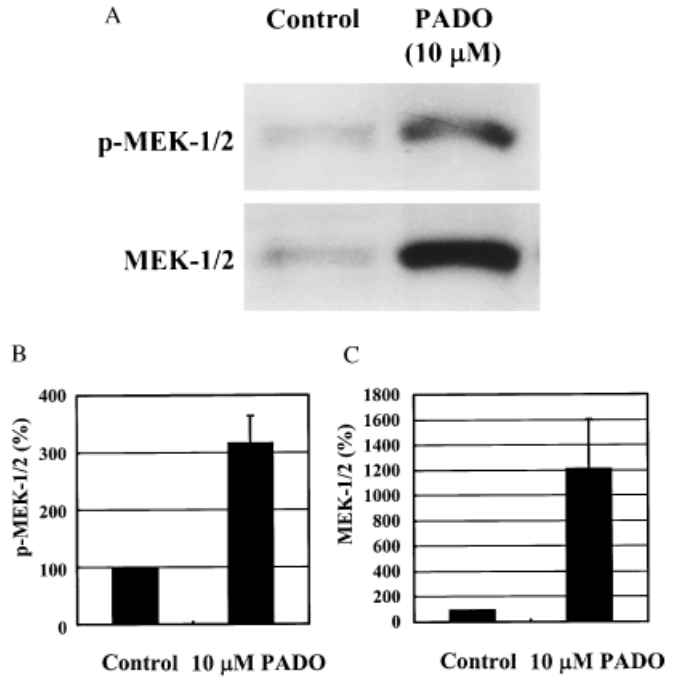

Figure 6. L-α-phosphatidic acid dramatically activates MEK-1/2 and upregulates the level of MEK-1/2 in cultured murine hair epithelial cells. Western blotting analytical results are shown for p-MEK-1/2 and MEK-1/2 extracted from cultured murine hair epithelial cells. Ten micromoles of L-α-phosphatidic acid dioleoyl (PADO) was added to the culture medium during the final 96 h of the 7 d culture period. L-α-phosphatidic acid dioleoyl (10 μM) dissolved in purified water was added at a rate of 1% (vol/vol) to the culture medium. (*A*) The data show the control for p-MEK-1/2, 10 μM L-α-phosphatidic acid dioleoyl for p-MEK-1/2, control for MEK-1/2, and 10 μM L-α-phosphatidic acid dioleoyl for MEK-1/2. Specific immunoreactive 45 kDa band for p-MEK-1/2 and MEK-1/2 was detected. Typical results are shown for three independent experiments performed. (*B*), (*C*) Quantitative analysis was performed by densitometry. The level of p-MEK-1/2 (*B*) or MEK-1/2 (*C*) in the controls is represented as 100. Values are represented as mean ± SD of three independent experiments.

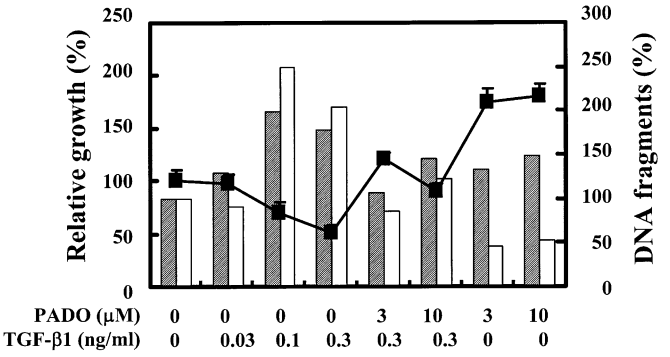

Figure 7. TGF-β1 concentration-dependently decreases hair epithelial cell growth and induces apoptosis in hair epithelial cells; addition of L-α-phosphatidic acid dioleoyl to the culture counteracts the inhibitory effect of TGF-β1. The effects of TGF-β1 and phosphatidic acid on hair epithelial cells were monitored. The growth-promoting activities (■) for hair epithelial cells relative to controls (=100%) are shown. Apoptotic cell death was quantitatively measured by detecting mononucleosomes and oligonucleosomes in both the cell lysate (*hatched bar*) and the culture medium supernatant (*clear bar*) by ELISA assay. DNA fragments in both the cell lysate and the culture medium supernatant relative to controls (=100%) are shown. TGF-β1 and L-α-phosphatidic acid dioleoyl (PADO) dissolved in purified water was added at a volume of 1/100 to the culture medium in the final 2 d of the 5 d culture period. For the control, medium without TGF-β1 or L-α-phosphatidic acid dioleoyl was used.

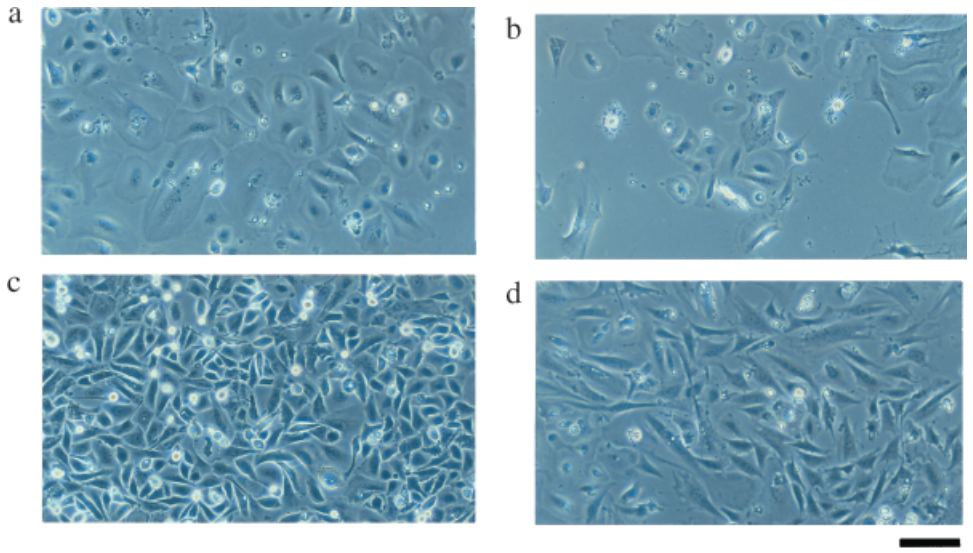

Figure 8. Micrographs of hair epithelial cells cultured in the presence of TGF-β1 and L-α-phosphatidic acid dioleoyl. Hair epithelial cells cultured for 5 d are shown. (*a*) Control of cultured murine hair epithelial cells; (*b*) murine hair epithelial cells cultured in the presence of 0.3 ng per ml of TGF-β1; (*c*) murine hair epithelial cells cultured in the presence of 10 μM L–phosphatidic acid dioleoyl; (*d*) murine hair epithelial cells cultured in the presence of both 0.3 ng per ml of TGF-1 and 10 μM L–phosphatidic acid dioleoyl. *Scale bars*: 100 μm.

effects have been reported on epidermal keratinocytes: phosphatidic acid has been shown to exert an antiapoptotic effect on epidermal keratinocytes exposed to oxidative stress caused by hydrogen peroxide (Lee *et al*, 2000b), which is known to mediate mitochondrial-dependent apoptosis induced by TGF-β (Herrera *et al*, 2001). The MAPK signaling pathway, which is the signal transduction route affected by phosphatidic acid, was identified through its interaction with GPCR, Ras, or Raf-1. On the other hand, it has been reported that activation of MAPKK (= MEK) upregulates the expression of Bcl-x_L (Jost *et al*, 2001), an antiapoptotic factor (Stoll *et al*, 1998). We confirmed that phosphatidic acid activates MEK in hair epithelial cells (**Fig 6**). These pieces of information, taken together, suggest that the hair-growing mechanism of phosphatidic acid is at least partially based on its inhibitory effects on TGF-β-induced apoptotic cell death in hair epithelial cells through MAPK activation stimulated by a phosphatidic-acid-related signal transduction pathway.

We speculate that the hair-growing activity of phosphatidic acid is at least linked to its growth-promoting effect on hair epithelial cells and its protective action on TGF-β1-induced apoptotic cell death that is assumed to trigger catagen induction (Seiberg *et al*, 1995; Foitzik *et al*, 2000) in the hair cycle. Both mechanisms are speculated to be linked to activation of the MAPK signaling pathway.

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