J. Biochem. **134**, 441–445 (2003)
DOI: 10.1093/jb/mvg162

Role of p38 MAPK in Lupeol-Induced B16 2F2 Mouse Melanoma Cell Differentiation

Keishi Hata, Kazuyuki Hori* and Saori Takahashi

Department of Bioengineering, Akita Research Institute of Food & Brewing (ARIF), 4-26 Sanuki, Araya-machi, Akita 010-1623

Received June 24, 2003; accepted July 9, 2003

We examined the signaling mechanisms involved in the differentiation-inducing activity of lupeol toward B16 2F2 melanoma cells. α-Melanocyte stimulating hormone (α-MSH), forskolin and dibutyryl cAMP, which are believed to be cAMP-elevating agents and analogues, enhanced lupeol-induced B16 2F2 cell differentiation. However, H89, an inhibitor of protein kinase A, completely abolished B16–2F2 cell differentiation induced by lupeol. Furthermore, we studied the role of mitogen-activated protein kinases (MAPKs) in lupeol-induced B16 2F2 cell differentiation. U0126, an inhibitor of MAPK kinases, induced B16 2F2 cell differentiation and enhanced the cell differentiation induced by lupeol. However, SB203580, a selective inhibitor of p38 MAPK, completely blocked lupeol-induced B16 2F2 cell differentiation. Western blot analysis revealed that 10 μM lupeol transiently elevated the level of phosphorylation of p38 MAPK. The phosphorylation of p38 MAPK was detected on the addition of 1 μM lupeone, another lupane triterpene, but was not induced by 1 μM lupeol. These results suggested that lupeol induced B16 2F2 cell differentiation through activation of p38 MAPK, and that the structural differences at C-3 of lupane triterpenes played an important role in the activation of p38 MAPK.

Key words: B16 2F2 cell, cAMP, differentiation, lupeol, p38 MAPK, relationship, structure–activity.

Abbreviations: db cAMP, dibutyryl cAMP; ERK, extracellular signal-regulated protein kinase; MAPK, mitogen-activated protein kinase; MEK, MAPK/ERK kinase; α-MSH, α-melanocyte stimulating hormone; PKA, protein kinase A; PKC, protein kinase C.

Mouse melanoma cell line B16 was isolated from C57 BL/6 mice and is known to produce melanin pigments (*1*). Some reports have suggested that up-regulation of melanogenesis was induced through B16 cell differentiation, and cAMP-elevating agents [α-melanocyte stimulating hormone (α-MSH), isobutylmethylxanthine and forskolin] (*2*), inhibitors of phosphatidylinositol 3-kinase/p70^{S6}-kinase (LY294002 and rapamycin) (*3*), and mannosylerythritol lipid (MEL) (*4*) were found to induce B16 cell differentiation. The signaling mechanisms of these inducers of melanocyte/melanoma cell differentiation were studied, and it was indicated that the activation of protein kinase C (PKC) was required for melanocyte/melanoma cell pigmentation (*5–7*). Additionally, cAMP, through protein kinase A (PKA), induced functional and morphological differentiation of melanocyte/melanoma cells (*8–10*). Some studies have indicated that the extracellular signal-regulated kinase (ERK) 1/2 pathway was responsible for high levels in cell proliferation and differentiation of many cancers (*11*, *12*). Signaling in the ERK 1/2 pathway up-regulated the melanin synthesis in melanocyte/melanoma cells by regulating PKA or PKC downstream (*13–15*). Recently, some studies revealed that the ERK 1/2 signal was not required for the melanogenesis in B16 and other melanoma cells (*16*, *17*). More recently, the p38 mitogen-activated protein kinase (MAPK) cascade was demonstrated to be involved in the melanogenesis in B16 induced by α-MSH (*18*).

We previously isolated lupeol from Chinese dandelion roots (hokouei-kon), and it was found to induce B16 2F2 cell differentiation (*19*). In a study on the structure-activity relationship of lupane triterpenes, we showed that the keto function at C-3 of lupane triterpenes enhanced the differentiation-inducing activities of lupane triterpenes toward B16 2F2 cells, and that a carbonyl group at C-17 was essential for the apoptosis-inducing activities toward the cells (*20*). Furthermore, we showed that the lupane triterpenes with a carbonyl group at C-17 inhibited human topoisomerase I *in vitro*, and that the inhibitory effect might trigger the induction of the apoptosis of some types of cancer cell (*21*). In the present study, we examined the effect of lupeol on the signaling mechanism involved in B16 2F2 cell differentiation, and showed that the activation of p38 MAPK played an important role in the B16 2F2 cell differentiation induced by lupane triterpenes. Moreover, we examined the structure-activity relationship of lupane triterpenes as to their activation of p38 MAPK.

*To whom correspondence should be addressed. Tel: +81-18-888-2000, Fax: +81-88-2008, E-mail: hori@arif.pref.akita.jp

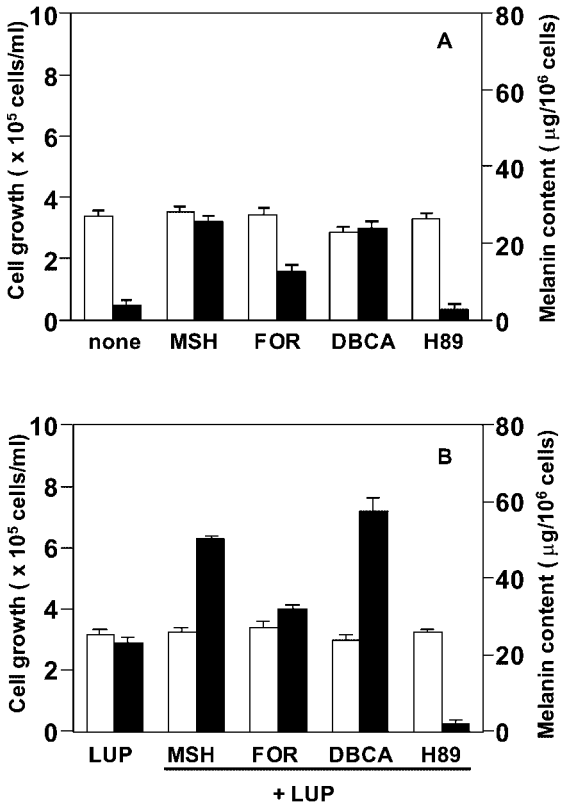

Fig. 1. **Effects of adenyl cyclase activators and a PKA inhibitor.** Following treatment of B16 2F2 cells with 5 μM α-MSH (MSH), 10 μM forskolin (FOR), 1 mM db-cAMP (DBCA), or 5 μM H89, in the absence (A) or presence (B) of 10 μM lupeol, the cell growth (white bars) and melanin content (black bars) were measured.

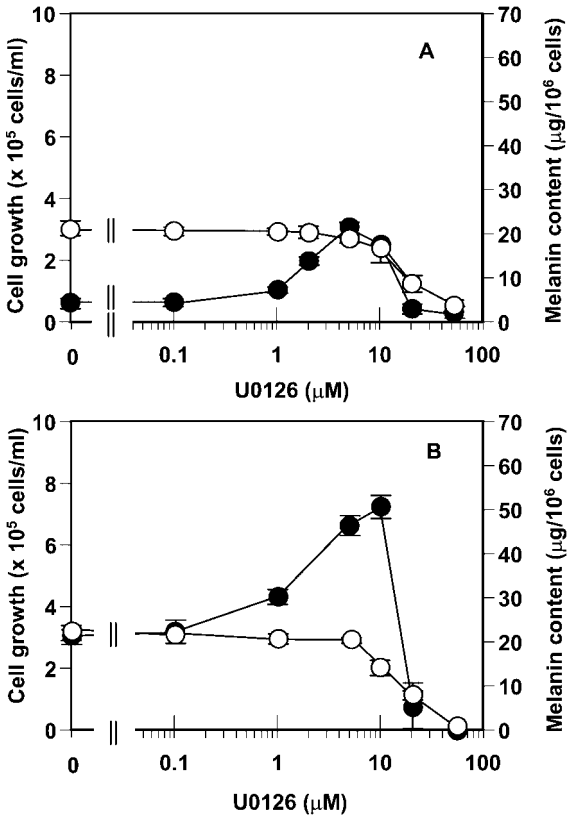

Fig. 2. **Synergistic effects of U0126 and lupeol on the melanogenesis in B16 2F2 cells.** Following treatment of B16 2F2 cells with various concentrations of U0126 in the absence (A) or presence (B) 10 μM lupeol for 72 h, the cell growth (open circles) and melanin content (solid circles) were measured.

MATERIALS AND METHODS

Materials—α-MSH, anti-p38 MAPK, db cAMP, forskolin, PD98059, and SB203580 were purchased from Sigma Chemical (St. Louis, MO). Anti-active p38 MAPK, alkaline phosphatase-conjugated anti-rabbit IgG (H+L), 5-bromo-4-chloro-3-indoyl-phosphate/nitroblue tetrazolium (BCIP/ NBT) color development substrate, and U0126 were from PROMEGA (Madison, WI).

Cell Culture and General Method—B16 2F2 cells (*19*) were maintained in Dulbecco's modified Eagle's medium containing 10% FBS, 100 μg/ml streptomycin and 100 U/ml penicillin.

Effects of PKA Activators or an Inhibitor—Aliquots of 1ml of B16 2F2 cells (1×10^5 cells) were treated with α-MSH (5 μM), forskolin (10 μM), db-cAMP (1 mM), or H89 (5 μM) in the absence or presence of 10 μM lupeol for 72h. The cell growth and melanin content were measured as described previously (*19*).

Effects of Inhibitors of MAPK Pathways—Aliquots of 1 ml of B16 2F2 cells (1×10^5 cells) were treated with U0126 (0.1–10 μM) or SB203580 (1–10 μM) in the presence or absence of 10 μM lupeol for 72 h, and then the cell growth and melanin content were measured.

Western Blot Analysis—Aliquots of 10 ml of B16 2F2 cells (1×10^5 cells/ml) were incubated with or without a stimulant for an appropriate time. The cells were collected by pipetting, washed with PBS twice, and then lysed with 5 mM Trs-HCl buffer, pH6.8, containing 1% SDS, 1 mM EDTA and 10% sucrose (100 μl). The proteins (50 μg) were separated by SDS polyacrylamide gel electrophoresis (*22*) using a 5–20% gel (PAGEL 520N; ATTO) and then transferred to a nitrocellulose membrane. The membrane was immersed in 20 mM Tris-HCl, pH 7.5, 0.15 M NaCl, 0.05% Tween 20 (TBST) containing 5% skim milk, and then incubated with anti-ERK 1/2 antibodies (1:5,000 dilution with TBST), anti-phospho-ERK 1/2 antibodies (1:5,000 dilution with TBST), anti-p38 MAPK antibodies (1:10,000 dilution with TBST) or anti-phospho-p38 MAPK antibodies (1:2,000 dilution with TBST) for 2 h at room temperature, washed three times with TBST, and then incubated with alkaline phosphatase-conjugated anti-rabbit IgG (Fc) (1:5,000 dilution with TBST) for 1 h at room temperature. The membrane was washed three times with TBST, and then allowed to react with BCIP/NBT for color development.

RESULTS

Effects of PKA Activators and an Inhibitor—Briefly, we examined the effects of α-MSH and forskolin (activators of adenyl cyclase), db-cAMP (a cAMP analogue), and H89 (an inhibitor of PKA) on lupeol-induced B16 2F2 cell differentiation. With 5 μM α-MSH, 10 μM forskolin or 1 mM db-cAMP, melanin synthesis in B16 2F2 cells was induced 7.4-, 3.6- and 6.8-fold, respectively, when com-

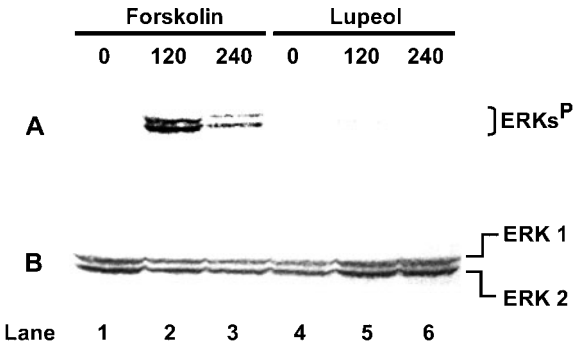

Fig. 3. **Effects of forskolin and lupeol on the activation of ERK1/2.** Following treatment of B16 2F2 cells with 10 μM forskolin (lanes 1–3) or 10 μM lupeol (lanes 4–6) for the indicated periods, cell lysates were analyzed by Western blotting using anti-phospho ERK 1/2 (ERKs^P, A) antibodies and anti-ERK 1/2 (B) antibodies.

pared to untreated B16 2F2 cells (Fig. 1A). Lupeol at 10 μM up-regulated the melanin synthesis 6.6-fold (Fig. 1B). Three PKA activators enhanced the up-regulation of B16 2F2 cell melanogenesis induced by 10 μM lupeol, *i.e.* 2.2-, 1.4-, and 2.5-fold, respectively, when compared to B16 2F2 cells stimulated with lupeol. Moreover, 5 μM H89 completely blocked the stimulatory effect of lupeol on the melanin synthesis in B16 2F2 cells.

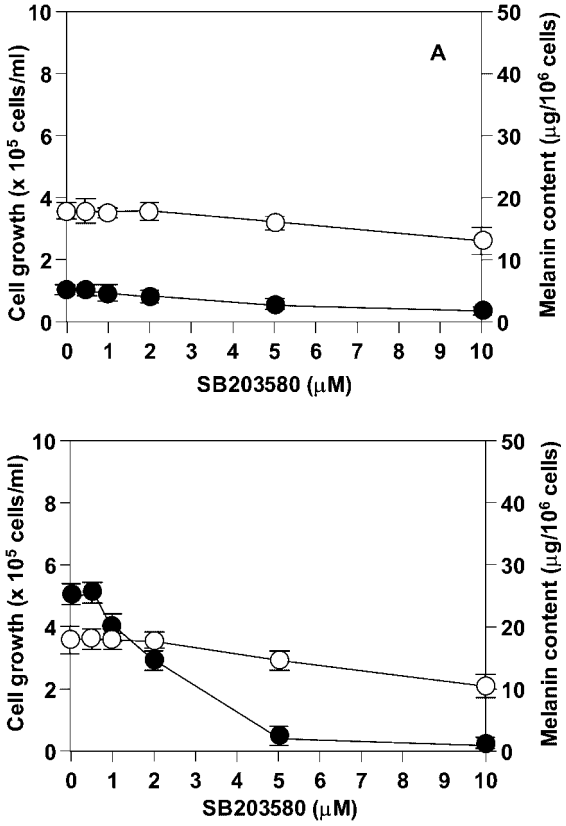

Fig. 4. **Inhibitory effects of SB203580 on lupeol-induced B16 2F2 cell differentiation.** B16 2F2 cells were incubated with various concentrations of SB203580 in the absence (A) or presence of (B) 10 μM lupeol for 72 h, and then the cell growth (open circles) and melanin content (solid circles) were measured.

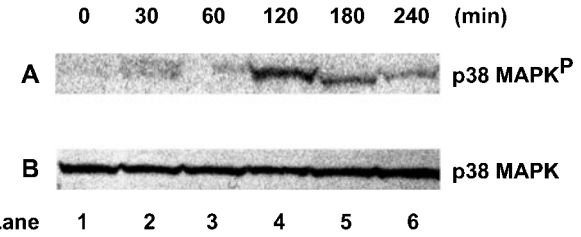

Fig. 5. **Activation of p38 MAPK by lupeol.** B16 2F2 cells were incubated with 10 μM lupeol for 0–240 min (lanes 1–6), and then cell lysates were analyzed by Western blotting using anti-phospho p38 MAPK (p38 MAPK^P) antibodies (A) or anti-p38 MAPK antibodies (B).

Effect of U0126—Figure 2 shows the effects of U0126, an inhibitor of MAPK/ERK kinase (MEK) 1/2, on the melanin synthesis in B16 2F2 cells in the absence and presence of 10 μM lupeol. U0126 at between 2 and 10 μM induced B16 2F2 cell differentiation. The melanin synthesis in B16 2F2 cells was increased 5.8-fold by the addition of 5 μM U0126, when compared to untreated B16 2F2 cells (Fig. 2A). Furthermore, U0126 enhanced lupeol-induced B16 cell differentiation. The melanin synthesis in B16 2F2 cells treated with 5 and 10 μM U0126 in the presence of 10 μM lupeol was increased 2.2- and 2.3-fold, respectively, when compared to B16 2F2 cells stimulated with lupeol alone (Fig. 2B). We then investigated the effect of lupeol and forskolin on the activation of ERK 1/2 by Western blot analysis (Fig. 3). ERK 1/2 in B16 2F2 cells was activated on treatment with 10 μM forskolin for 120 min and 240 min (Fig. 3A, lanes 2 and 3). However, 10 μM lupeol did not activate ERK 1/2 (lanes 4–6).

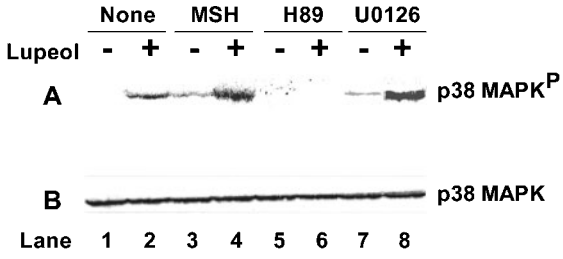

Fig. 6. **Effects of melanogenesis regulators on the activation of p38 MAPK.** B16 2F2 cells were untreated (lane 1) or treated with 10 μM lupeol (lane 2), 5 μM α-MSH (lane 3), 10 μM lupeol plus 5 μM α-MSH (lane 4), 5 μM H89 (lane 5), 10 μM lupeol plus 5 μM H89 (lane 6), 5 μM U0126 (lane 7), or 10 μM lupeol plus 5 μM U0126 (lane 8) for 120 min. The proteins in cell lysates were separated by SDS-PAGE, followed by Western blot analysis with anti-phospho p38 MAPK antibodies (A) or anti-p38 MAPK antibodies (B).

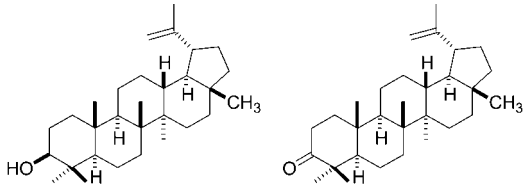

Fig. 7. **Chemical structures of lupeol and lupeone.**

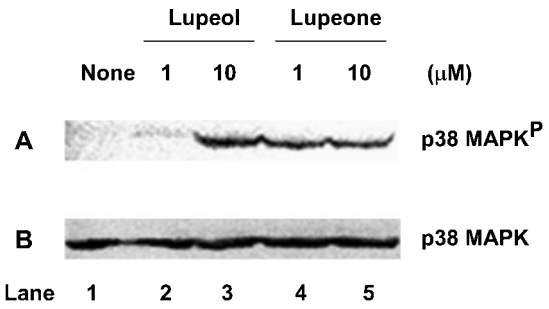

Fig. 8. **Structure–activity relationships of lupane triterpenes as to the activation of p38 MAPK.** B16 2F2 cells were incubated without (lane 1), or with 1 μM lupeol (lane 2), 10 μM lupeol (lane 3), 1 μM lupeone (lane 4), or 10 μM lupeone (lane 5) for 120 min. The proteins in cell lysates were separated by SDS-PAGE, followed by Western blot analysis with anti-phospho p38 MAPK antibodies (A) or anti- p38 MAPK antibodies (B).

Role of p38 MAPK in Lupeol-Induced B16 2F2 Cell Differentiation—We examined the effect of SB203580, an inhibitor of p38 MAPK, on lupeol-induced B16 2F2 cell differentiation (Fig. 4). SB203580 at 5 μM decreased the melanin biosynthesis in B16 2F2 cells 0.47-fold compared with untreated cells (Fig. 4A). Furthermore, SB203580 inhibited the up-regulation of melanin synthesis in B16 2F2 cells by lupeol in a dose-dependent manner, and the up-regulation induced by 10 μM lupeol was completely abolished by 5 μM SB203580 (Fig. 4B). Therefore, we examined the level of phospho-p38 MAPK in lupeol-treated B16 2F2 cells by Western blotting (Fig. 5). The level of p38 MAPK in B16 2F2 cells was constant with 10 μM lupeol for 240 min (Fig. 6B). However, the phosphorylation of p38 MAPK was induced by 10 μM lupeol for 120 min, and then the level decreased (Fig. 6A).

Effects of Melanogenesis Regulators on the Activation of p38 MAPK by Lupeol—We studied the effects of α-MSH, U0126, and H89 on the phosphorylation of p38 MAPK in B16 2F2 cells by lupeol (Fig. 6). α-MSH and U0126, which up-regulated B16 2F2 cell melanogenesis, induced the phosphorylation of p38 MAPK in B16 2F2 cells (Fig. 6A lanes 3 and 7, respectively), and accelerated the phosphorylation of p38 MAPK induced by lupeol (Fig. 6A, lanes 4 and 8, respectively). H89, a blocker of lupeol-induced B16 2F2 cell differentiation, did not induce the phosphorylation of p38 MAPK, and completely abolished the lupeol-induced phosphorylation of p38 MAPK (Fig. 6A, lanes 4 and 5, respectively).

Structure-Activity Relationship of the Activation of p38 MAPK by Lupane Triterpenes—In our previous study (*20*), lupeone, another lupane triterpene (Fig. 7), markedly induced B16 2F2 cell melanogenesis at lower concentrations than with lupeol. The effects of the two lupane triterpenes on the level of phospho-p38 MAPK in B16 2F2 cells were investigated (Fig. 8). Phosphorylation of p38 MAPK increased with 10 μM lupeol (Fig. 8A, lane 3), when compared to untreated B16 2F2 cells (Fig. 8A, lane 1), but the level of phospho-p38 MAPK in 1 μM lupeol-treated B16 2F2 cells was very low (Fig. 8A, lane 2). However, phospho-p38 MAPK was detected in B16 2F2 cells treated with 1 and 10 μM lupeone (Fig. 8A, lanes 4 and 5, respectively).

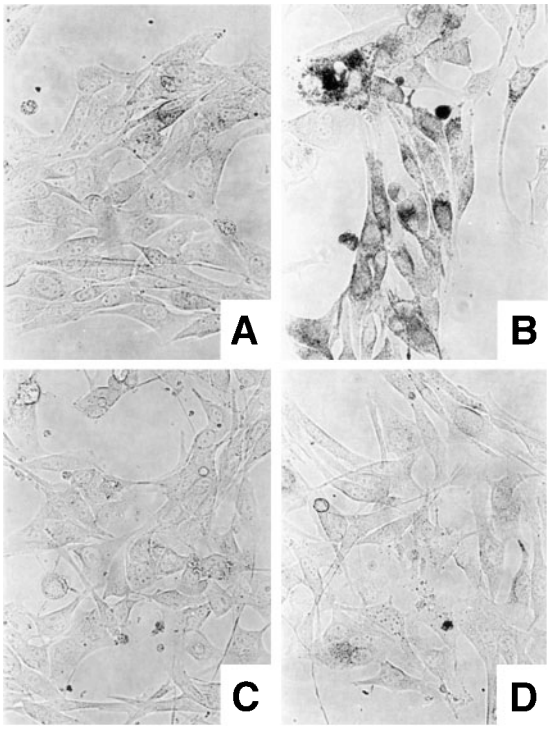

Fig. 9. **Photomicrographs of B16 2F2 cells treated without (A), or with 10 μM lupeol (B), 10 μM lupeol plus 5 μM H89 (C), or 10 μM lupeol plus 5 μM SB203580 (D) for 72 h.**

DISCUSSION

Previously, lupeol was found to exhibit differentiation-inducing activity toward B16 2F2 cells. On B16 2F2 cell differentiation induced by lupeol, the intracellular melanin content markedly increased (Fig. 9, A and B). In the present study, we investigated the signaling mechanism of lupeol as to B16 2F2 cell differentiation. cAMP-elevating agents such as α-MSH and forskolin induce the pigmentation of melanocyte/melanoma cells through the PKA pathway (*2*, *8*). Recently, it was reported that histamine induced tyrosinase expression in human melanocytes, and H89, a PKA inhibitor, blocked the expression of tyrosinase, a key enzyme in melanogenesis, in human melanocytes (*10*). Therefore, we examined the effects of cAMP- elevating agents or H89 on the up-regulation of melanogenesis in B16 2F2 cells by lupeol. cAMP-elevating agents and a cAMP analogue (db cAMP) enhanced the stimulatory effects of lupeol on the melanin synthesis in B16 2F2 cells. H89 completely abolished the differentiation-inducing activity of lupeol toward B16 2F2 cells (Fig. 9C), thus indicating positive involvement of the cAMP and PKA pathways in lupeol-induced B16 2F2 cell differentiation. Activation of ERK 1/2 was found in differentiation of some cancer cells, and PD98059, an inhibitor of MEK 1, blocked the differentiation phenotype of neuroblastoma and leukemia cells (*11*, *12*). It has been reported that the inhibition of MEK 1 by PD98059 and a negative dominant mutant of MEK led to the induction of B16 melanoma cell melanogenesis (*16*). We showed that the addition of U0126, a selective inhibitor of MEK 1/2, induced B16 2F2 cell differentiation and had a synergis-

tic effect on the B16 2F2 melanogenesis stimulated by lupeol. Forskolin is known to activate ERK1/2 in melanocyte/melanoma cells (*23*). We studied the effect of lupeol on the activation of ERK 1/2 using forskolin as a positive activator. It was revealed that ERK 1/2 was activated in B16 2F2 cells incubated with 10 μM forskolin for 120–240 min, but that lupeol did not activate the ERK 1/2 pathway. These results suggested that the signaling in the ERK 1/2 pathway was not required for lupeol-induced B16 2F2 differentiation. Recently, the activation of p38 MAPK was demonstrated to trigger the B16 cell differentiation induced by α-MSH (*18*). Therefore, we studied the effect of lupeol on the activation of p38 MAPK in B16 2F2 cells. SB203580, a selective inhibitor of p38 MAPK, completely blocked the melanin synthesis in B16 2F2 cells stimulated by lupeol (Fig. 9D). Additionally, Western blot analysis revealed that lupeol transiently induced the phosphorylation of p38 MAPK in B16 2F2 cells. Furthermore, α-MSH and U0126 increased the level of phospho-p38 MAPK stimulation by lupeol, and H89 completely blocked the phosphorylation of p38 MAPK induced by lupeol. We concluded that the p38 MAPK pathway was involved in lupeol-induced B16 2F2 cell differentiation as a downstream target of PKA.

Previously, we demonstrated that different moieties of lupane triterpenes separately regulated their differentiation- and apoptosis-inducing activities toward B16 2F2 cells. Lupane triterpenes that had a carbonyl group at C-17 induced the apoptosis of some types of human cancers through inhibition of human topoisomerase I (*20*, *21*). In the present study, we showed that 1 μM lupeone induced the phosphorylation of p38 MAPK, whereas the same concentration of lupeol did not activate p38 MAPK. This finding agreed with the results of studies on the relationship between the structures of lupane triterpenes and their differentiation-inducing activities toward B16 2F2 cells.

This work was supported in part by a research grant from the Ministry of Education, Culture, Sports, Science and Technology of Japan.

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