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Induction of tissue inhibitor of matrix metalloproteinase-2 by cholesterol depletion leads to the conversion of proMMP-2 into active MMP-2 in human dermal fibroblasts

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Abbreviations: Cav-1, caveolin-1; MMP-2, matrix metalloproteinase-2; MβCD, methyl-β-cyclodextrin; TIMP-2, tissue inhibitor of matrix metalloproteinase-2

Abstract

Cholesterol is one of major components of cell membrane and plays a role in vesicular trafficking and cellular signaling. We investigated the effects of cholesterol on matrix metalloproteinase-2 (MMP-2) activation in human dermal fibroblasts. We found that tissue inhibitor of matrix metalloproteinase-2 (TIMP-2) expression and active form MMP-2 (64 kD) were dose-dependently increased by methyl-β-cyclodextrin (MβCD), a cholesterol depletion agent. In contrast, cholesterol depletion-induced TIMP-2 expression and MMP-2 activation were suppressed by cholesterol repletion. Then we investigated the regulatory mechanism of TIMP-2 expression by cholesterol depletion. We found that the phosphorylation of JNK as well as ERK was significantly increased by cholesterol depletion. Moreover, cholesterol depletion-induced TIMP-2 expression and MMP-2 activation was significantly decreased by MEK inhibitor U0126, and JNK inhibitor SP600125, respectively. While a low dose of recombinant TIMP-2 (100 ng/ml) increased the level of active MMP-2 (64 kD), the high dose of TIMP-2 ($\geq$ 200 ng/ml) decreased the level of active MMP-2 (64 kD). Taken together, we suggest that the induction of TIMP-2 by cholesterol depletion leads to the conversion of proMMP-2 (72 kD) into active MMP-2 (64 kD) in human dermal fibroblasts.

Keywords: cholesterol; extracellular signal-regulated MAP kinases; JNK mitogen-activated protein kinases; matrix metalloproteinase 2; tissue inhibitor of metalloproteinase-2

Introduction

Cholesterol is one of the major components of skin lipid and synthesized in epidermal keratinocytes (Ponec *et al*., 1985). Cholesterol, a product of the isoprenoid/mevalonate pathway, is essential for cellular functions, such as cell growth and differentiation (Goldstein and Brown, 1990; Schmidt *et al*., 1991). Cellular cholesterol, of which most (up to 90 %) resides in the plasma membrane, is crucial for normal membrane permeability and fluidity and also plays a role in cellular signaling (Lange, 1991). However, it has not been fully elucidated the function of cholesterol on involvement of tissue inhibitor of matrix metalloproteinase-2 (TIMP-2) expression and matrix metalloproteinase-2 (MMP-2) activation which are causing genes of skin aging. Therefore, in this study, we investigated the effects of cholesterol on TIMP-2 expression and MMP-2 activation in human dermal fibroblasts.

Matrix metalloproteinases (MMPs) are a family of zinc-dependent metalloendopeptidases collectively capable of degrading essentially all extracellular matrix (ECM) and are known to be involved in tissue remodeling, and angiogenesis (Rittie and Fisher, 2002; Kerkela and Saarialho-Kere, 2003). MMP-2 catalyzes the destruction of ECM such as type IV collagen and gelatin (Liotta, 1986; Woessner, 1991). The activation of proMMP-2 is regulated by a complex mechanism involving formation of a trimolecular complex with MT1-MMP (membrane type I-matrix metalloproteinase) and

TIMP-2 (Ellerbroek and Stack, 1999). TIMP-2 plays a dual role in the regulation of MMP-2 activation. TIMP-2 bridges the interaction between adjacent TIMP-2-free MT1-MMP and proMMP-2 (72 kD), activating proMMP-2 (72 kD) at low concentration, while further increase of TIMP-2 generates complete inhibition of this reaction (Strongin *et al*., 1995; Itoh *et al*., 1998; Ellerbroek and Stack, 1999). Meanwhile, it has been known that TIMP-2 expression is regulated by ERK and p38 pathway. A TGF-β1-induced TIMP-2 expression was decreased by inhibition of ERK1/2, while it is sustained with the treatment of p38 MAPK inhibitor (Munshi *et al*., 2004). However, the relationship between JNK and TIMP-2 has not been reported yet.

In this study, we confirmed the activation of MMP-2 is mediated by TIMP-2, and we found this mechanism can be controlled according to the different concentration of cholesterol. In human dermal fibroblasts, depletion of cholesterol increases the phosphorylation of MAPK, not only ERK but also JNK, resulted in TIMP-2 induction. When we treated MEK1/2 or JNK specific inhibitors, the expression of TIMP-2 was significantly decreased. Conclusively, TIMP-2 induction by cholesterol depletion causes the conversion of proMMP-2 (72 kD) in active MMP-2 (64 kD) in human dermal fibroblasts. JNK as well as ERK may mediate this mechanism.

Results

TIMP-2 expression and MMP-2 activation are increased by cholesterol depletion in human dermal fibroblasts

To determine the effect of cholesterol on TIMP-2 expression and MMP-2 activation in human dermal fibroblasts, we treated the cells with the indicated concentration of cholesterol depletion agent, methyl β cyclodextrin (MβCD), for 1 h and then cultured for 72 h in fresh serum-free media. TIMP-2 expression in culture media was dose-dependently increased by cholesterol depletion (Figure 1A). The expression of TIMP-2 was increased significantly by 551 ± 19% and 560 ± 38% of control level with 0.5% and 1% of MβCD treatment, respectively.

To investigate the effect of cholesterol depletion on MMP-2 activation, we treated the cells with cholesterol depletion agent, MβCD, in human dermal fibroblasts for 1 h at the indicated concentration. After 72 h, MMP-2 activation by MβCD was observed in culture media using zymography. Our results revealed that cholesterol

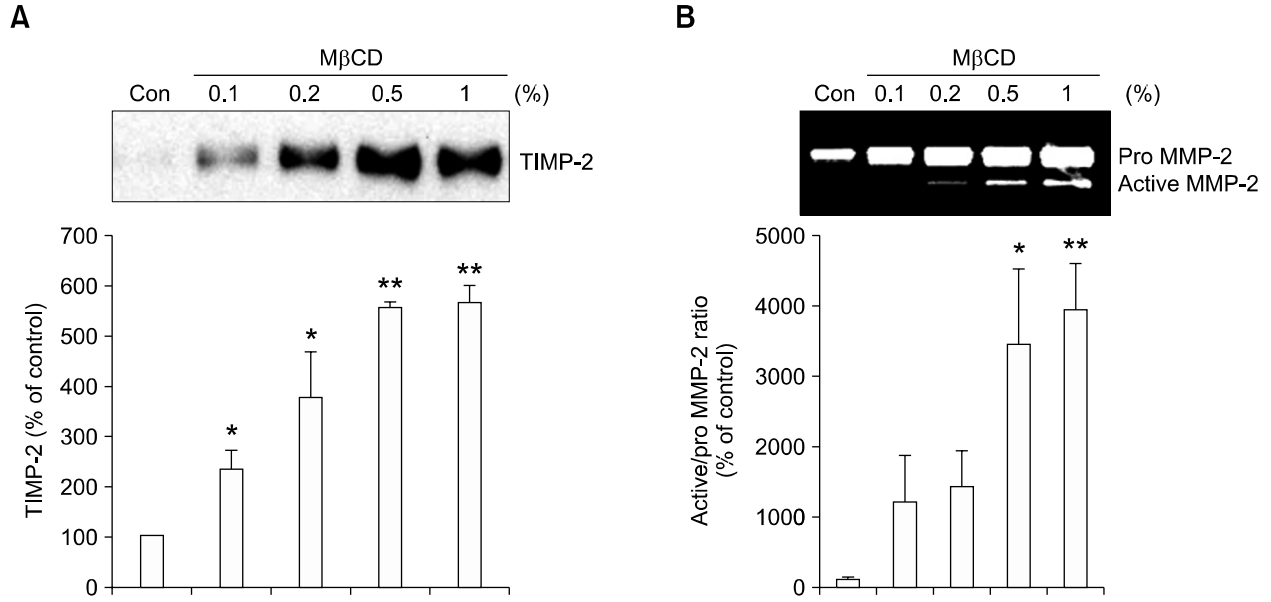

Figure 1. TIMP-2 expression and MMP-2 activation are increased by cholesterol depletion in human dermal fibroblasts. (A, B) After serum-starvation for 24 h, cells were treated with cholesterol depletion agent, MβCD, at the indicated concentrations for 1 h and then further incubated for 72 h. TIMP-2 protein expression and MMP-2 activation were determined by Western blotting (A) and zymography (B), respectively. The results shown are representative of three independent experiments. Values shown are means ± SEM. **P* < 0.05, ***P* < 0.01 vs. Con, control.

depletion by MβCD dose-dependently increased active form of MMP-2 (64 kD) (Figure 1B). Stimulation of cells with MβCD increased the ratio of active MMP-2 (64 kD) to proMMP-2 (72 kD) activity by the average of 3,420 ± 1,120% and 3,880 ± 721% of control level at the concentration of 0.5% and 1% MβCD, respectively.

The amount of intracellular cholesterol was significantly decreased by 50% of control level after 1% MβCD treatment for 30 min (Figure 2A). To confirm the morphology of plasma membrane by cholesterol depletion, we observed the caveolae structure using the electron microscopy. As shown in Figure 2B, caveolae structure was disappeared by cholesterol depletion. In addition, we found the interaction between MT1-MMP and caveolin-1 by immunoprecipitation (Figure 2C). This result is evidence that MMP-2 activation events happened in caveolae of plasma membrane.

Cholesterol depletion-induced TIMP-2 expression and MMP-2 activation are decreased by cholesterol repletion in human dermal fibroblasts

On the other hand, the effect of cholesterol repletion on cholesterol depletion-induced TIMP-2 expression was investigated in human dermal fibroblasts. The cells were treated with 1% MβCD with or without 100 μg/ml cholesterol for 1 h, and then further incubated 72 h in fresh serum-free media. Cholesterol depletion-induced TIMP-2 expression was significantly prevented by cholesterol treatment (Figure 3A).

In addition, we also investigated the effects of cholesterol repletion on cholesterol depletion-induced MMP-2 activation. Cholesterol depletion-induced MMP-2 activation is significantly decreased by cholesterol (100 μg/ml) treatment (Figure 3B). The ratio of active MMP-2 (64 kD) to proMMP-2 (72 kD) activity by cholesterol depletion was significantly increased by 3,503 ± 1,020% of control level, whereas cholesterol depletion-induced MMP-2 activation was decreased to 1,144 ± 290% of control level by the treatment of 100 μg/ml cholesterol.

Under the same condition, the amount of intracellular cholesterol was significantly decreased by MβCD treatment, while it was reversed by cholesterol repletion (Figure 3C). Thus, we suggest that TIMP-2 expression is regulated by cholesterol in human dermal fibroblasts.

Cholesterol depletion-induced TIMP-2 expression is mediated by ERK and JNK-dependent pathways in human dermal fibroblasts

To investigate the regulatory mechanisms involved in TIMP-2 expression by cholesterol depletion in

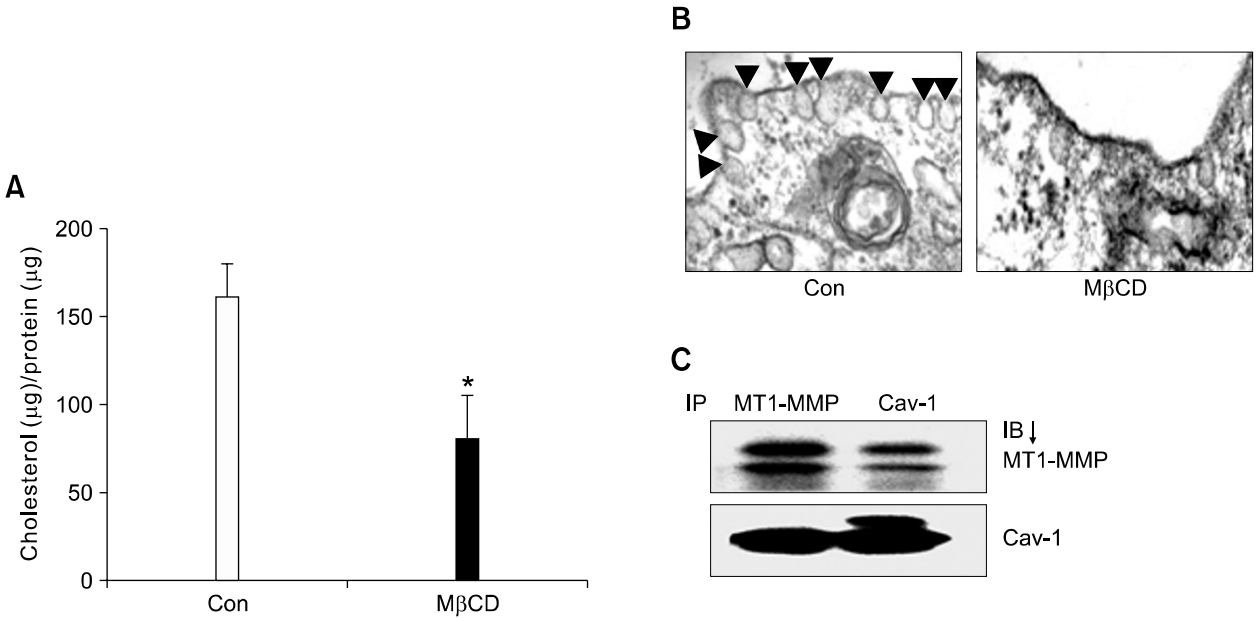

Figure 2. The amount of intracellular cholesterol level and caveolae structure in human dermal fibroblasts. (A) Cholesterol assays described in Methods. (B) The structure of plasma membrane by MβCD treatment. The *arrow head* indicates caveolae structures. Bar = 100 nm. (C) Total cell lysates were immunoprecipitated with anti-caveolin-1 and MT1-MMP antibodies. Immunocomplexes were immobilized on protein A-agarose, washed three times with lysis buffer, separated by 12% SDS-PAGE gel, and immunoblotted with anti-caveolin-1 and anti-MT1-MMP antibodies, respectively. The results shown are representative of three independent experiments. Values shown are means ± SEM. *$P < 0.05$, vs. control. Con, control.

human dermal fibroblasts, we observed the effects of cholesterol depletion on the activation of MAP kinases, including ERK and JNK. Cells were treated with 1% MβCD for the indicated times. Cholesterol depletion by MβCD treatment increased phosphorylation of ERK and JNK in human dermal fibroblasts (Figure 4). ERK and JNK phosphorylation peaked at 15 min after MβCD treatment. However, the phosphorylation of p38 kinase tended to decrease with cholesterol depletion by MβCD (data not shown).

Then, we examined the effects of MAPK specific inhibitors, including MEK inhibitor (U0126) and JNK inhibitor (SP600125) on cholesterol depletion-induced TIMP-2 expression in human dermal fibroblasts. Cells were pretreated with each inhibitor for 30 min and then further incubated with 1% MβCD for 1 h. Inhibition of ERK and JNK pathways by U0126 and SP600125, respectively, suppressed cholesterol depletion-induced TIMP-2 expression (Figure 5A). These data suggest that induction of TIMP-2 expression by cholesterol depletion may be mediated by activation of ERK- and JNK-dependent pathways in human dermal

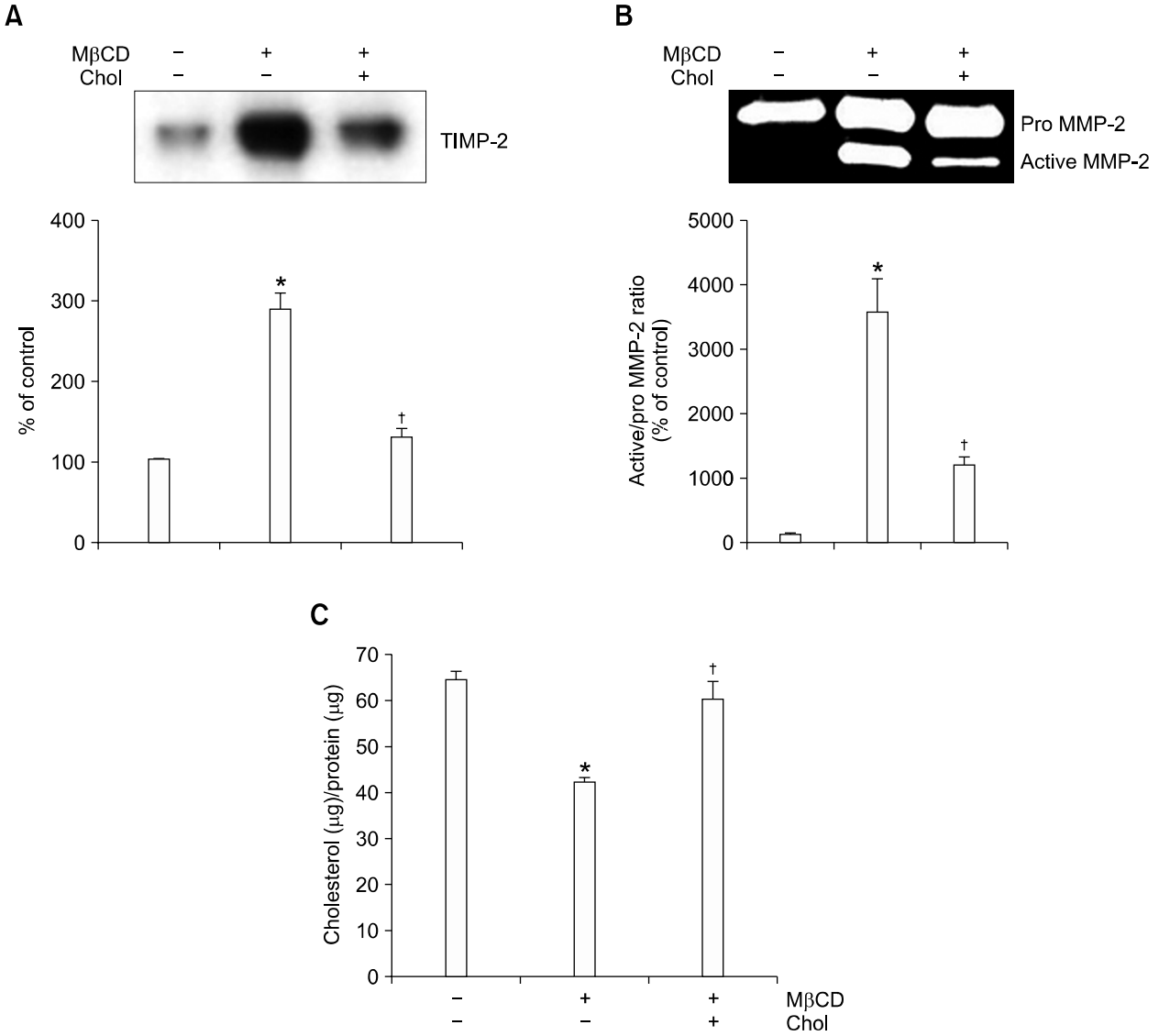

Figure 3. Cholesterol depletion-induced TIMP-2 expression and MMP-2 activation are decreased by cholesterol repletion in human dermal fibroblasts. (A, B) After serum-starvation for 24 h, cells were treated with 1% MβCD and/or 100 mg/ml cholesterol for 1 h and further cultured 72 h. TIMP-2 protein expression and MMP-2 activation were determined by Western blotting (A) and zymography (B), respectively. (C) Cholesterol assays described in Methods. The results shown are representative of three independent experiments. Values shown are means ± SEM. *$P < 0.05$ vs. control, †$P < 0.05$ vs. MβCD-treated cells. Con, control; Chol, cholesterol.

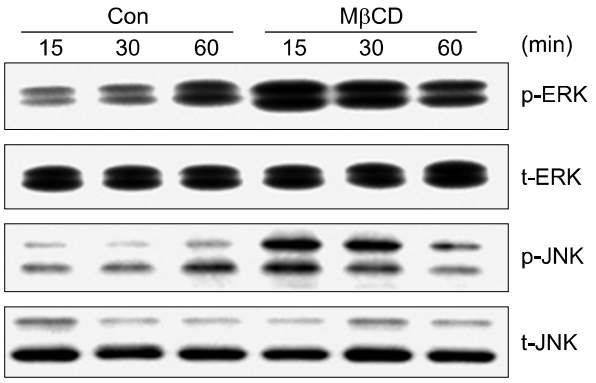

Figure 4. The phosphorylation of ERK and JNK is increased by cholesterol depletion in human dermal fibroblasts. After serum-starved for 24 h, cells were treated with 1% MβCD and further incubated at 37°C for the indicated times. Total- and phospho-ERK and JNK were measured in whole cell lysates by Western blotting. The results shown are representative of three independent experiments. Con, control.

fibroblasts.

Next, we examined the effects of MAPK specific inhibitors, including U0126 and SP600125 on cholesterol depletion-induced MMP-2 activation in human dermal fibroblasts. Cells were pretreated with each inhibitor for 30 min and then further incubated with 1% MβCD for 1 h. Cholesterol depletion-induced MMP-2 activation is inhibited by U0126 and SP600125, respectively (Figure 5B). These results indicated that cholesterol level may lead to proMMP-2 into active MMP-2 through ERK and JNK-dependent pathways in human dermal fibroblasts.

To investigate the effect of TIMP-2 on MMP-2 activation, we treated purified TIMP-2 protein to the culture media of human dermal fibroblasts for 72 h. Our results showed that low concentration of TIMP-2 (100 ng/ml) treatment greatly increased MMP-2 activity, while high concentration of TIMP-2 ($\geq$ 400 ng/ml) treatment decreased the gelatinase activity of MMP-2 in human dermal fibroblasts (Figure 5C). These results suggest that cholesterol depletion-induced TIMP-2 expression may mediate MMP-2 activation. Thus, we suggest that cholesterol depletion-induced TIMP-2 expression play an important role in the conversion of proMMP-2 (72 kD) into active MMP-2 (64 kD) in human dermal fibroblasts.

Discussion

Chronologically aged skin exhibits delayed recovery rates after defined barrier insults, decreased epidermal lipid synthesis, and reduced cholesterol synthesis in particular (Zettersten *et al*., 1997). UV irradiation triggers the alteration of skin lipid component including cholesterol reduction and ceramide induction (Elyassaki and Wu, 2006; Kim *et al*., 2007, 2008).

MMP-2 specifically cleaves type IV collagen, the major component of the basement membrane (Nelson *et al*., 2000). In addition, MMP-2 is higher in the dermis of photoaged skin than naturally aged skin and MMP-2 tends to be higher in aged and photoaged skin than in young skin (Chung *et al*., 2001). 14-3-3ε significantly increases the transcriptional activity of MMP-2 gene in NIH3T3 cells (Lee *et al*., 2009). However, it has not been well known the function of cholesterol on MMP-2 activation in human skin. Therefore, we investigated the function of cholesterol on MMP-2 activation with human dermal fibroblasts.

It has been reported that the addition of titrated amount of TIMP-2 stimulated the conversion of proMMP-2 into the activated form, while further increase of TIMP-2 concentration caused complete inhibition of the reaction (Strongin *et al*., 1993, 1995). Consistent with these results, we showed that TIMP-2 expression was greatly increased by cholesterol depletion with MβCD. On the other hand, treatment of cholesterol suppressed cholesterol depletion-induced TIMP-2 expression. Also, treatment of purified human TIMP-2 increased the level of active MMP-2 at low concentration (100 ng/ml), while high concentration of TIMP-2 ($\geq$ 400 ng/ml) decreased MMP-2 activity. Therefore, we demonstrated that induction of TIMP-2 expression by cholesterol depletion might directly regulate MMP-2 activation in human dermal fibroblasts.

Previously, it was reported that cholesterol depletion activates MMP-2 in HT1080 fibrosarcoma cells, though the increase of the amount of MT1-MMP on the cell surface (Atkinson *et al*., 2004). Our results, in accordance to this finding, showed that MMP-2 activation is increased by cholesterol depletion, while it is decreased by cholesterol repletion. However, we could not find any meaningful alteration of MT1-MMP expression by cholesterol depletion in human dermal fibroblasts (data not shown). On the other hand, we found TIMP-2 expression was greatly increased by cholesterol depletion induced by MβCD, resulted in MMP-2 activation. Therefore, we suggest that MMP-2 activation by cholesterol depletion is related to the induction of TIMP-2 rather than MT1-MMP.

Degradation and remodeling of the ECM and basement membranes by MMPs are essential steps in skin aging process. In particular, TIMP-2 can form a complex with MT1-MMP, which then serves as a receptor for proMMP-2 (Strongin *et al*.,

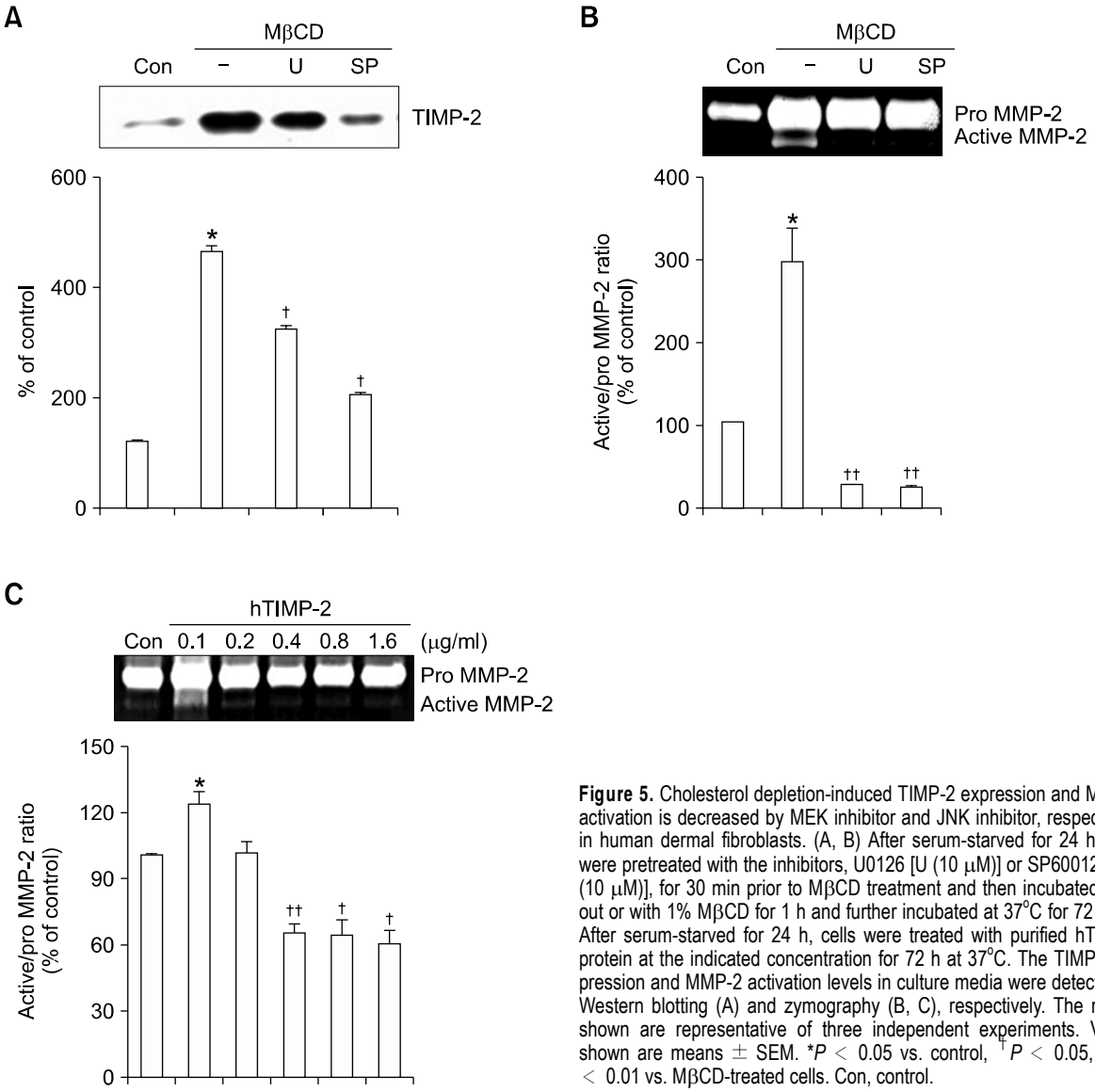

Figure 5. Cholesterol depletion-induced TIMP-2 expression and MMP-2 activation is decreased by MEK inhibitor and JNK inhibitor, respectively, in human dermal fibroblasts. (A, B) After serum-starved for 24 h, cells were pretreated with the inhibitors, U0126 [U (10 μM)] or SP600125 [SP (10 μM)], for 30 min prior to MβCD treatment and then incubated without or with 1% MβCD for 1 h and further incubated at 37°C for 72 h. (C) After serum-starved for 24 h, cells were treated with purified hTIMP-2 protein at the indicated concentration for 72 h at 37°C. The TIMP-2 expression and MMP-2 activation levels in culture media were detected by Western blotting (A) and zymography (B, C), respectively. The results shown are representative of three independent experiments. Values shown are means ± SEM. *$P < 0.05$ vs. control, †$P < 0.05$, ††$P < 0.01$ vs. MβCD-treated cells. Con, control.

1995). TIMP-2 plays an important role on activation of proMMP-2. Hence, low concentrations of TIMP-2 promote processing of MMP-2 to its proteolytically active form, but high concentrations of TIMP-2 inhibit MMP-2 activation (Ellerbroek and Stack, 1999). Therefore, we suggest that the balance between proMMP-2 and TIMP-2 may affect directly the degradation of ECM during skin aging process.

Both MMP-2 and MT1-MMP can be localized in specialized plasma membrane extensions (Monsky *et al*., 1993). MMP-2 colocalizes with caveolae on the surface of endothelial cells (Puyraimond *et al*., 2001) and MT1-MMP is highly enriched in low-density Triton X-100-insoluble membrane domains that contain the caveolar marker protein, caveolin-1 (Annabi *et al*., 2001). Interestingly, we found that caveolin-1 directly binds with MT1-MMP by co-immunoprecipitation. Based on this, it may be postulated that the events of MMP-2 activation by cholesterol depletion may happen in the caveolae, which is a specialized microdomain of plasma membrane.

In previous studies, expression of a constitutively active MEK1 mutant increased the promoter activity of TIMP-2, whereas expression of a constitutively active MKK3 decreased the promoter activity of TIMP-2 (Munshi *et al*., 2004). The TIMP-2 promoter contains AP-1, AP-2, and SP-1 binding sites which can potentially be modulated

by ERK1/2 and p38 MAPKs (Hammani *et al*., 1996). Interestingly, we also observed that phosphorylation of ERK as well as JNK is increased by cholesterol depletion. Cholesterol depletion-induced TIMP-2 expression and MMP-2 activation are significantly decreased by MEK inhibitor, U0126, and JNK inhibitor, SP600125, respectively. Based on this, we suggest that cholesterol may regulate phosphorylation of ERK and JNK and then, induce TIMP-2 expression, resulting in MMP-2 activation in human dermal fibroblasts.

In conclusion, we demonstrated that the intracellular level of cholesterol regulates phosphorylation of MAPK such as ERK and JNK. These pathways mediate TIMP-2 induction and then, trigger MMP-2 activation in human dermal fibroblasts. Increase of cholesterol levels may be a good strategy to prevent MMP-2 activation-mediated degradation of ECM in human skin.

Methods

Cell cultures

Primary human dermal fibroblasts obtained from foreskin of healthy volunteers, age 3-12 y. The skin was minced, followed by incubation with collagenase (1 mg/ml in DMEM) for 1-2 h at 37°C. Collagenase was then removed by washing with DMEM (Life Technology, Rockville, Maryland). The isolated cells were allowed to attach on plastic plates and cultured in DMEM supplemented with 10% FBS (Hyclone, Logan, Utah), 2 mM glutamine, 100 IU/ml penicillin, and 100 μg/ml streptomycin. After six to eight passages, the fibroblasts were used for experiments.

MβCD and cholesterol treatments

For experiments, fibroblasts were maintained in culture medium without FBS for 24 h and then, 1% MβCD (Sigma, St.Louis, MO) and/or 100 μg/ml cholesterol (Sigma, St.Louis, MO) were added for 1 h. Culture media were replaced with fresh media without FBS and the cells were further incubated for the indicated times. In experiments involving MAPK inhibitors including U0126 and SP600125 (Calbiochem, San Diego, CA), these were added 30 min prior to 1% MβCD and/or 100 μg/ml cholesterol. Three independent normal human dermal fibroblasts cells were used in this study.

Electron microscopic analysis

Human dermal fibroblasts with or without cholesterol depletion agent, MβCD were pelleted and fixed with 3% glutaraldehyde/PBS saline (pH 7.4). After washing with 0.2 M sodium cacodylate buffer (pH 7.4), the cell pellets were treated with 1% osmium tetroxide in cacodylate buffer for 1 h. Cells were then dehydrated in graded ethanol through propylene oxide and embedded in epoxy resin (Polyscience Co.). Ultrathin sections were cut and stained with uranyl acetate and lead citrate. Sections were observed using a transmission electron microscope (H-600, Hitachi).

Measurement of cellular cholesterol contents

Serum starved human dermal fibroblasts were treated with or without 1% MβCD and/or 100 μg/ml cholesterol for 1 h. At the end of the incubation, the cells were washed and scraped. The pellet was extracted with 2:1 methanol:chloroform, followed by 0.5 ml of chloroform. The chloroform (lipid phase) was separated and evaporated in a SpeedVac. The lipid was solubilized in 1 ml of the cholesterol assay kit (ShinYang, Seoul, Korea) buffer solution. Samples were incubated for 1 h at 37°C prior to measuring absorbance at 505 nm.

Immunoprecipitation

Cells were washed with ice-cold PBS, and lysed in a buffer containing 20 mM Tris–HCl, pH 7.4, 300 mM NaCl, 2 mM EDTA, 2 mM EGTA, pH 8.0, 0.4 mM sodium vanadate, 0.4 mM PMSF, and 2% Triton X-100. The cell lysates were centrifuged at 12,000 rpm for 15 min at 4°C and the supernatants were subjected to immunoprecipitation with either anti-caveolin-1 (Santa Cruz Biotechnology, Santa Cruz, CA) or anti-MT1-MMP antibodies (Chemicon International Inc., Temecula, CA) (1-5 μg/ml each). The immunocomplexes were absorbed to ProteinA/G PLUS-Agarose (Santa Cruz Biotechnology, Santa Cruz, CA) and washed three times with lysis buffer. After extensive washing, samples were resolved by the addition of 2× sample buffer, separated by 10% SDS-PAGE gel, and transferred to PVDF membranes. Blots were then probed by immunoblot analysis with anti-caveolin-1 (1:5,000 dilution), anti-MT1-MMP (1:1,000 dilution).

Immunoblotting

Cells were lysed with lysis buffer [150 mM NaCl, 10 mM Tris, (pH7.4), 1 mM EDTA, 1 mM EGTA (pH8.0), 1% Triton X-100, 0.5% NP-40, 0.2 mM PMSF, protease cocktail, phosphatase cocktail] and lysates were clarified by centrifugation at 12,000 rpm for 15 min at 4°C. Extracted protein was quantified by the Bradford assay and were separated by 10% SDS-PAGE and then transferred to PVDF membranes (Amersham Bioscience, Buckinghamshire, UK). After transfer, PVDF membranes were blocked with TBS containing 5% skim milk and incubated overnight at 4°C with individual antibodies against total- and phospho-ERK1/2, or JNK antibodies (Cell Signaling Technology, Beverly, MA). These membrane were washed and then, incubated with HRP-conjugated rabbit anti-mouse antibody or donkey anti-rabbit antibody (Santa Cruz Biotechnology, Santa Cruz, CA), respectively. The ECL western blotting system (Amersham Bioscience, Buckinghamshire, UK) was used as suggested for protein detection. Signal strengths were quantified using a densitometric program (Bio 1D; Vilber Lourmat, Marne La Vallec, France).

Zymography

To determine the activity of secreted MMP-2 in the culture media, zymography was performed in 10% zymogram gels (Invitrogen, Carsbad, CA) that were cast to include gelatin as previously described (Kim *et al*., 2009). Briefly, samples (50 μl) were suspended in loading buffer, and were run without prior denaturation on a 10% SDS-PAGE gels containing 0.5 mg/ml gelatin. After electrophoresis, gels were washed to remove SDS and incubated for 30 min at room temperature (RT) in a renaturing buffer (50 mM Tris, 5 mM $CaCl_2$, 0.02% NaN_3, 1% Triton X-100). The gels were then incubated for 24 h at 37°C in a developing buffer [50 mM Tris-HCl (pH7.8) 5 mM $CaCl_2$, 0.15 M NaCl, and 1% Triton X-100]. Proteolytic bands were visualized by staining with 0.5% Coomassie Brilliant Blue solution. Signal strengths were quantified using a densitometric program (Bio 1D; Vilber Lourmat, Marne La Vallec, France).

Acknowledgments

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