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Low-dose UVB irradiation stimulates matrix metalloproteinase-1 expression *via* a BLT2-linked pathway in HaCaT cells

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Abbreviations: 12(S)-HETE, 12(S)-hydroxyeicosatetraenoic acid; 12-LO, 12-lipoxygenase; BLT2, a low-affinity leukotriene B_4 receptor; LTB_4, leukotriene B_4; MMP-1, matrix metalloproteinase-1; Nox1, NADPH oxidase 1; UVB, ultraviolet B

Abstract

Skin exposure to low-dose ultraviolet B (UVB) light up-regulates the expression of matrix metalloproteinase-1 (MMP-1), thus contributing to premature skin aging (photo-aging). Although cyclooxygenase-2 (COX-2) and its product, prostaglandin E_2 (PGE_2), have been associated with UVB-induced signaling to MMP expression, very little are known about the roles of lipoxygenases and their products, especially leukotriene B_4 (LTB_4) and 12(S)-hydroxyeicosatetraenoic acid (12(S)-HETE), in MMP-1 expression in skin keratinocytes. In the present study, we demonstrate that BLT2, a cell surface receptor for LTB_4 and 12(S)-HETE, plays a critical role in UVB-mediated MMP-1 upregulation in human HaCaT keratinocytes. Moreover, our results demonstrated that BLT2-mediated MMP-1 upregulation occurs through a signaling pathway dependent on reactive oxygen species (ROS) production and the subsequent stimulation of ERK. Blockage of BLT2 *via* siRNA knockdown or with the BLT2-antagonist LY255283 completely abolished the up-regulated expression of MMP-1 induced by low-dose UVB irradiation. Finally, when HaCaT cells were transiently transfected with a BLT2 expression plasmid, MMP-1 expression was significantly enhanced, along with ERK phosphorylation, suggesting that BLT2 overexpression alone is sufficient for MMP-1 up-regulation. Together, our results suggest that the BLT2-ROS-ERK-linked cascade is a novel signaling mechanism for MMP-1 upregulation in low-dose UVB- irradiated keratinocytes and thus potentially contributes to photo-aging.

Keywords: 12-hydroxy-5,8,10,14-eicosatetraenoic acid; leukotriene B_4; LTB_4R2 protein, human; matrix metalloproteinase 1; reactive oxygen species; skin aging; ultraviolet rays

Introduction

Ultraviolet B (UVB) light is absorbed into the epidermis and causes various skin disorders. Whereas high-dose UVB irradiation causes apoptotic (e.g., sunburn) (Faustin and Reed, 2008; Yoshizumi *et al*., 2008; Ryu *et al*., 2010) or cancerous (Matsumura and Ananthaswamy, 2004; El-Abaseri *et al*., 2005) phenotypes in skin, low-dose UVB exposure has been suggested to accelerate skin aging or photo-aging, which includes major phenotypes such as wrinkles and sagging (Jenkins, 2002). The expression of matrix metalloproteinases (MMPs), which degrade cutaneous proteins (e.g., collagen and gelatin), is upregulated by low-dose UVB irradiation, resulting in a loss of elasticity and increased wrinkle formation in dermal tissue (Brenneisen *et al*., 2002).

MMPs can be classified into several groups including collagenase, gelatinase, stromelysin and membrane-type MMPs. MMP expression in normal tissues appears to be tightly regulated and is induced by various factors such as cytokines, growth factors, tumor promoters, oxidative stresses, heat-shock, and UV irradiation (Matrisian, 1994; Sternlicht and Werb, 2001). Collagen is a major structural component of the skin and maintenance of its level is important for skin elasticity. Among the various MMPs, MMP-1 is one of the important players in UVB-induced skin aging *via* collagen degradation (Kim *et al*., 2005, 2009). UV exposure leads to inflammatory responses in dermal skin (Hruza and Pentland, 1993; Katiyar *et al*., 1999; Pillai *et al*., 2005). Additionally, pro-inflammatory lipid mediators, such as prostaglandins (PGs) and leukotrienes (LTs), are inducibly synthesized following UV irradiation of dermal skin (Isoherranen *et al*., 1999; Yan *et al*., 2006). While many reports indicate that cyclooxygenase-2

(COX-2) and its product, prostaglandin E_2 (PGE_2), are associated with UVB irradiation-induced MMP expression in human skin (Isoherranen *et al*., 1999; Seo *et al*., 2003), little is known about the roles of lipoxygenase (LO)-derived lipid mediators in mediating MMP upregulation in UVB-irradiated skin. Recently, several groups have reported that the induced activity and/or expression of 5-lipoxygenase (5-LO) results in remarkable elevation of leukotriene B_4 (LTB_4) (Yan *et al*., 2006) and the induction of 12-lipoxygenase (12-LO), which is followed by elevated synthesis of 12(S)-hydroxyeicosatetraenoic acid (12(S)-HETE) (Rhodes *et al*., 2009) in the skin after UV irradiation.

LTB_4 is derived from arachidonic acid (AA) by the sequential actions of cytosolic phospholipase A_2 ($cPLA_2$), 5-LO, and leukotriene A_4 (LTA_4) hydrolase and is recognized by two kinds of LTB_4 receptors, BLT1 and BLT2. Unlike BLT1, BLT2 is ubiquitously expressed in human tissues and has broad substrate specificity for several eicosanoids, including 12-HHT, 12(S)-HETE, 12(S)-HPETE, and 15(S)-HETE, as well as LTB_4 (Tager and Luster, 2003). Though it has been reported that BLT2 is highly expressed in mouse skin and causes sunburn damage in response to high-dose UVB irradiation or the itch-associated scratching of mouse skin in response to products of 12-LO (Iizuka *et al*., 2005; Kim *et al*., 2008; Ryu *et al*., 2010), the exact function of BLT2 in skin has yet to be determined. In particular, no clear physiological role of BLT2 has been characterized in low dose UVB-induced photo-aging, especially in relation to MMP-1 expression.

Therefore, the aim of the present study was to investigate whether BLT2 plays a role in low-dose UVB irradiation-induced MMP-1 upregulation. We found that low-dose UVB irradiation significantly upregulated MMP-1 expression in human keratinocyte HaCaT cells through a pathway dependent on BLT2 (a receptor for LTB_4 and 12(S)-HETE). We also demonstrated that in UVB-irradiated HaCaT cells, BLT2 mediates MMP-1 upregulation through a signaling pathway dependent on reactive oxygen species (ROS) production and the subsequent stimulation of extracellular-regulated kinase (ERK), two well-characterized mediators of UVB-induced MMP-1 expression (Whitmarsh and Davis, 1996; Wertz *et al*., 2004; Kim *et al*., 2005; Shin *et al*., 2008; Yang *et al*., 2009). In agreement with the proposed role of BLT2 as a mediator of UVB-induced MMP-1 expression, we observed that BLT2 overexpression markedly enhanced MMP-1 expression and ERK phosphorylation. Taken together, our results demonstrate that a BLT2-ROS-ERK-linked pathway plays a crucial role in low-dose UVB-induced MMP-1 expression in human keratinocytes and thus potentially contributes to skin photo-aging.

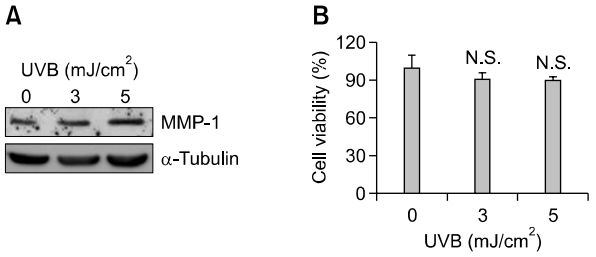

Figure 1. Low-dose UVB irradiation increases MMP-1 expression in HaCaT cells. (A) and (B), HaCaT cells were starved in serum-free DMEM for 24 h and then the cells were irradiated with increasing doses of UVB (0, 3, and 5 mJ/cm^2). (A) Levels of secreted MMP-1 in the culture medium were detected by western blotting 48 h after UVB irradiation; α-tubulin was used as a loading control. The results shown are representative of three independent experiments with similar results. (B) Cell viability was measured by the trypan blue exclusion assay 24 h after UVB irradiation. Data are the mean ± S.D. of three independent experiments (N.S., not significant).

Results

Low-dose UVB irradiation up-regulates MMP-1 expression in HaCaT cells

To examine the possible role of BLT2 in MMP-1 expression in response to UVB irradiation in HaCaT cells, we first tested whether MMP-1 expression is upregulated by low-dose UVB irradiation. To answer this question, we carried out western blotting using an equal volume of conditioned medium from UVB-irradiated cells (0, 3, and 5 mJ/cm^2; 48 h irradiation) and observed that MMP-1 expression was significantly increased by UVB exposure (Figure 1A). We performed trypan blue exclusion assay to measure cell viability and did not observe any cytotoxicity to cells under the low-dose UVB irradiation conditions ($\leq$ 5 mJ/cm^2, see Methods for details) (Figure 1B). However, significant cytotoxicity was detected with high-dose UVB irradiation ($\leq$ 10 mJ/cm^2) (data not shown). Together, these results suggest that a low dose (5 mJ/cm^2) of UVB irradiation markedly increases MMP-1 expression in HaCaT keratinocytes without affecting cell viability.

UVB-induced MMP-1 expression is regulated through a BLT2-cascade in HaCaT cells

Next, we tested whether BLT2 and its ligands, LTB_4 and 12(S)-HETE, play a role in low-dose UVB-induced MMP-1 expression in HaCaT cells. Ini-

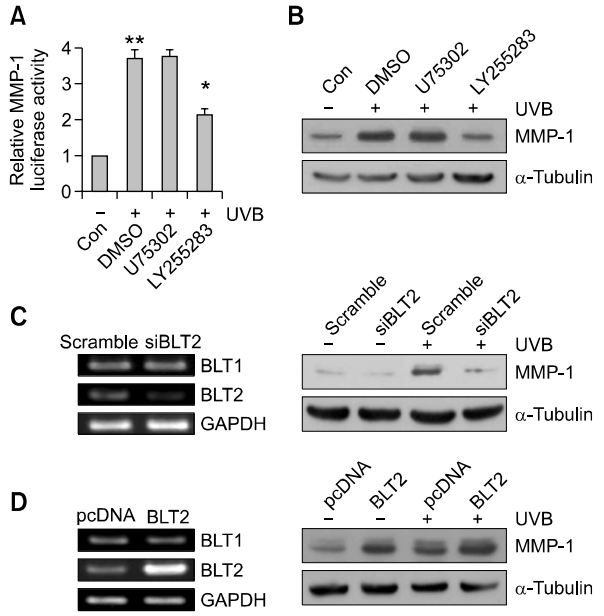

Figure 2. Low-dose UVB-induced MMP-1 expression is dependent on BLT2 in HaCaT cells. (A) HaCaT cells were transiently transfected with the MMP-1 reporter gene as described in Methods. The cells were starved in serum-free DMEM for 24 h. The cells were then incubated for 1 h with 1 μM U75302 or 10 μM LY255283 prior to UVB irradiation. Protein extracts were assayed for luciferase activity 24 h after UVB irradiation (5 mJ/cm^2). Data are the mean ± S.D. of three independent experiments (*, $P < 0.05$; **, $P < 0.01$). (B) HaCaT cells were starved in serum-free DMEM for 24 h. The cells were then incubated for 1 h with 1 μM U75302 or 10 μM LY255283 prior to UVB irradiation. Levels of secreted MMP-1 in the culture medium were detected by western blotting 48 h after UVB irradiation (5 mJ/cm^2). (C and D) HaCaT cells were transiently transfected with 50 μM BLT2-specific siRNA (C) or pcDNA-BLT2 (D) as described in Methods. The cells were then starved in serum-free DMEM for 24 h prior to UVB irradiation. Levels of secreted MMP-1 in the culture medium were detected by western blotting 48 h after UVB irradiation (5 mJ/cm^2); α-tubulin was used as a loading control. The results shown are representative of three independent experiments with similar results. To determine transfection efficiency, the total RNA was isolated, and the level of the BLT2 transcript was determined by semi-quantitative RT-PCR (C and D). The GAPDH level was used as a control.

tially, we examined the effect of a BLT2 antagonist (LY255283) on UVB-induced stimulation of MMP-1 promoter activity. As seen in Figure 2A, pretreatment with LY255283 remarkably suppressed UVB-stimulated MMP-1 promoter activity, whereas pretreatment with U75302, a BLT1 antagonist, had no effect. Similarly, western blotting analysis of the conditioned medium from UVB-irradiated cells showed that pretreatment with LY255283, but not U75302, clearly abolished the induced expression levels of MMP-1 (Figure 2B). Moreover, we observed that BLT2 knockdown with siRNA (siBLT2) completely suppressed UVB-induced MMP-1 expression (Figure 2C, right). These results suggest that BLT2 is crucial in mediating the low-dose UVB signaling of MMP-1 expression in HaCaT cells. Under the siBLT2 knockdown experimental conditions, the levels of BLT2, but not BLT1, were specifically reduced (Figure 2C, left). Additionally, we could observe a similar inhibitory effect by LY255283, but not by U75302, on UVB-induced MMP-1 expression in primary keratinocytes (data not shown), suggesting a potential common role of BLT2 in mediating MMP-1 expression in keratinocytes. Finally, to further demonstrate the role of BLT2 in mediating UVB-signaling of MMP-1 expression, we examined whether BLT2 overexpression is sufficient for inducing the expression of MMP-1. Thus, we transiently transfected HaCaT cells with the pcDNA-BLT2 expression plasmid. Under these conditions, elevated BLT2 mRNA levels were detected by semiquantitative RT-PCR analysis (Figure 2D, left). We clearly observed greatly enhanced MMP-1 expression through BLT2 overexpression alone (lane 2) compared to the control (Figure 2D, right). With UVB irradiation, further enhancement of MMP-1 expression was detected (Figure 2D, right). Therefore, these results suggest that BLT2 plays a critical mediatory role in UVB-induced MMP-1 expression in HaCaT cells.

UVB irradiation enhances the levels of BLT2 ligands and their synthesizing enzymes in HaCaT cells

To further elucidate the mediatory role of BLT2 in UVB-induced MMP-1 expression, we examined whether levels of the BLT2 ligands LTB_4 and 12(S)-HETE are also elevated in UVB-irradiated HaCaT cells. To explore this question, we performed specific enzyme-linked immunosorbent assays (ELISAs) to measure the levels of these ligands following UVB irradiation. Figure 3A shows that low-dose UVB irradiation greatly increased the production levels of LTB_4 and 12(S)-HETE. Next, we assessed the expression levels of 5- and 12-LO, which are enzymes responsible for the synthesis of LTB_4 or 12(S)-HETE, respectively. Figure 3B shows that 5- and 12-LO levels were also significantly upregulated following UVB irradiation. Blockage of LTB_4 and 12(S)-HETE synthesis with MK886 (5-LO/5-LO activating protein inhibitor) and Baicalein (12-LO inhibitor), respectively, was shown to diminish UVB-induced MMP-1 expression (Figure 3C). However, the addition of LTB_4 or 12(S)-HETE to the inhibitor-treated cells restored UVB-induced MMP-1 expression (Figure 3C). These results suggest that BLT2 signaling, along with augmented synthesis of its ligands LTB_4 and 12(S)-HETE, play roles in mediating MMP-1 expression in response to UVB irradiation in HaCaT cells.

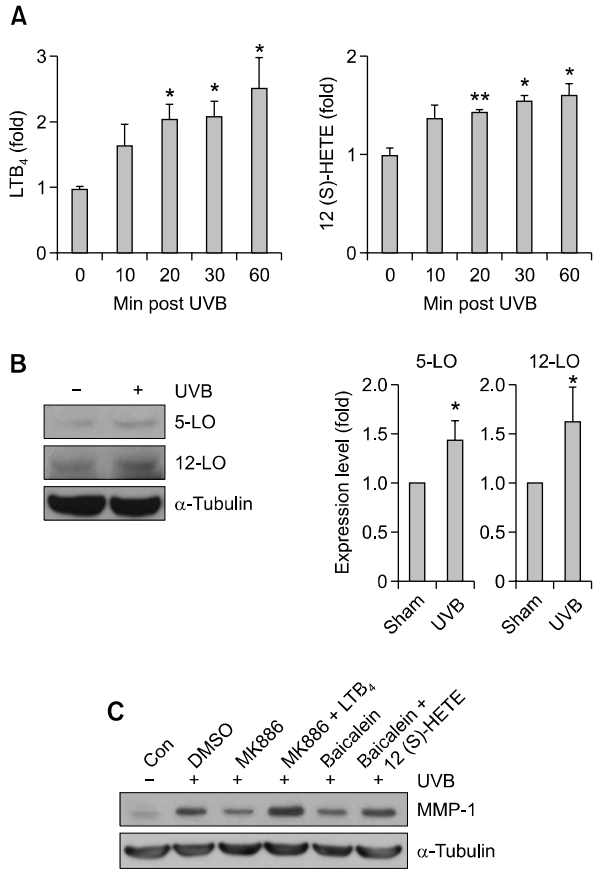

Figure 3. Low-dose UVB irradiation enhances the levels of BLT2 ligands and their synthesizing enzymes (5-LO and 12-LO) in HaCaT cells. (A) HaCaT cells were starved in serum-free DMEM for 24 h prior to UVB irradiation. The level of secreted LTB_4 or 12(S)-HETE in the culture medium was determined by specific ELISAs at the indicated times (0, 10, 20, 30, and 60 min) after UVB irradiation. Data are the mean ± S.D. (*, $P < 0.05$; **, $P < 0.01$; $n = 5$)(see Methods for details). (B) Proteins were extracted from cells irradiated with UVB (5 mJ/cm^2, 30 min) and the protein levels were western blotted using antibodies against 5- or 12-LO; α-tubulin was used as a loading control. The results shown are representative of three independent experiments with similar results. The expression levels of 5- and 12-LO were assessed using Student's t-test and expressed as mean ± S.D. (*, $P < 0.05$). (C) The cells were incubated for 1 h with 10 μM MK886 or 20 μM baicalein prior to UVB irradiation. Levels of secreted MMP-1 in the culture medium were detected by western blotting 48 h after UVB irradiation (5 mJ/cm^2); α-tubulin was used as a loading control.

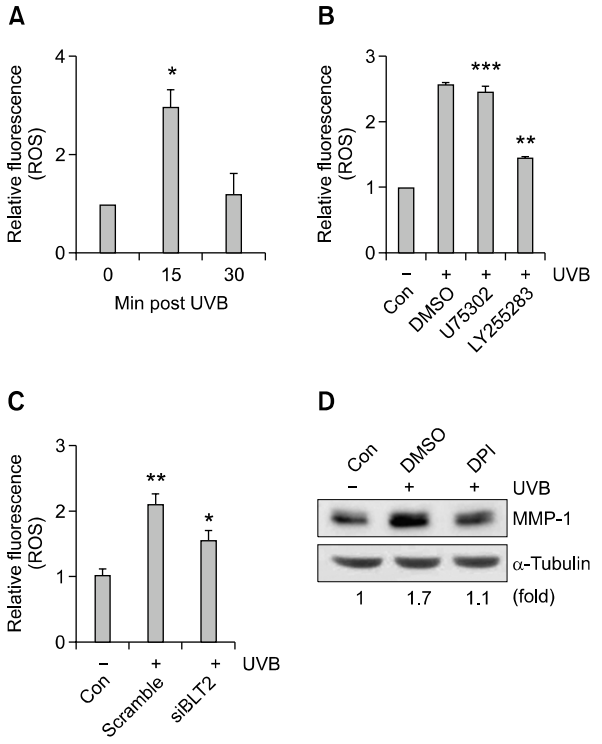

Figure 4. Low-dose UVB-induced MMP-1 expression is dependent on the BLT2-ROS-linked cascade. HaCaT cells were starved in serum-free DMEM for 24 h prior to UVB irradiation. The cells were incubated with 20 μM 2', 7'-dichlorofluorescein diacetate (DCFDA), an H_2O_2-sensitive fluorophore, for 20 min in the dark. Intracellular ROS levels were monitored by FACS analysis of DCF fluorescence after UVB irradiation (5 mJ/cm^2). (A) HaCaT cells were irradiated for the indicated times (0, 15, and 30 min) before ROS measurement. Also, cells were pretreated with inhibitors (1 μM U75302 or 10 μM LY255283) (B) for 1 h or transfected with 50 μM scrambled siRNA or siBLT2 for 24 h (C) prior to UVB irradiation. Then, the ROS levels were measured. All data are the mean ± S.D. of three independent experiments (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$). (D) The cells were incubated with 0.1 μM DPI for 1 h prior to UVB irradiation. Levels of secreted MMP-1 in the culture medium were detected by western blotting 48 h after UVB irradiation (5 mJ/cm^2); α-tubulin was used as a loading control. The results shown are representative of three independent experiments with similar results.

Low-dose UVB-induced MMP-1 expression is dependent on the BLT2-ROS-linked cascade

Accumulating evidence suggests that elevated ROS have mediatory roles in the expression of MMPs induced by UVB (Fisher *et al*., 1996; Whitmarsh and Davis, 1996; Brenneisen *et al*., 2002; Vayalil *et al*., 2004). Additionally, we previously reported that an LTB_4-BLT2 cascade is associated with the generation of ROS, which cause various cellular responses (Woo *et al*., 2002; Choi *et al*., 2008; Ryu *et al*., 2010). Therefore, we suspected that BLT2 may play a role in the generation of ROS triggered by low-dose UVB irradiation. To test this suspicion, ROS levels were monitored by FACS analysis at 0, 15, and 30 min after UVB irradiation (5 mJ/cm^2). Similar to previous reports, we observed that low-dose UVB irradiation rapidly increases ROS generation, reaching a maximum within 15 min (Figure 4A). Additionally, pretreatment with LY255283, but not U75302, significantly suppressed low-dose UVB-induced ROS generation (Figure 4B), suggesting a mediatory role for BLT2 in UVB-induced ROS generation. To further examine the role of BLT2 in ROS generation by low-dose UVB irradiation, we knocked down BLT2 with siBLT2 and similar

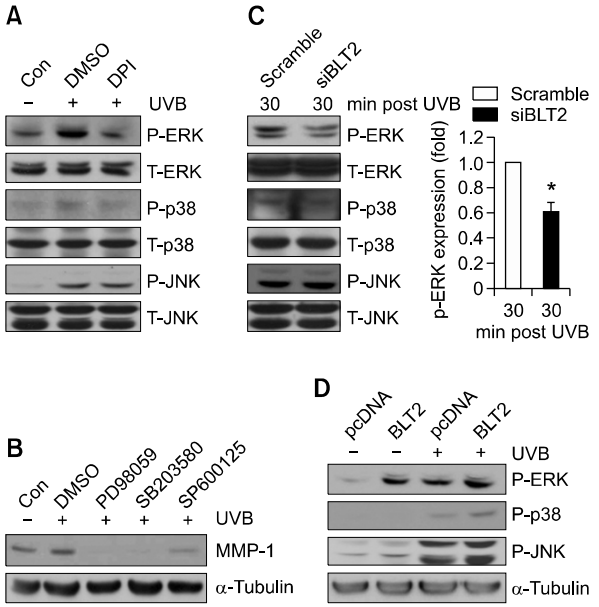

Figure 5. ERK phosphorylation lies downstream of the BLT2-ROS cascade in UVB signaling of MMP-1 expression in HaCaT cells. (A) After starvation for 24 h in serum-free DMEM, the cells were incubated with 0.1 μM DPI for 1 h prior to UVB irradiation. Proteins were extracted, western-blotted, and hybridized with antibodies against MAPKs (p-ERK, p-p38, and p-JNK) 30 min after UVB irradiation (5 mJ/cm^2). The results shown are representative of three independent experiments with similar results. (B) The cells were incubated with 20 μM PD98059, SB203580, or SP600125 for 1 h prior to UVB irradiation. Levels of secreted MMP-1 in the culture medium were detected by western blotting 48 h after UVB irradiation (5 mJ/cm^2); α-tubulin was used as a loading control. The results shown are representative of three independent experiments with similar results. (C) The cells were transiently transfected with 50 μM scrambled or BLT2-specific siRNA and then starved for 24 h in serum-free DMEM. Proteins were extracted and western blotted with antibodies against MAPKs (p-ERK, p-p38, and p-JNK) at 30 min after UVB irradiation. The levels of p-ERK were assessed using Student's t-test and expressed as mean ± S.D. of three independent experiments (*, $P < 0.05$). (D) HaCaT cells were transiently transfected with pcDNA or pcDNA-BLT2 for 24 h. After transfection, cells were starved in serum-free DMEM for 24 h prior to UVB irradiation. Proteins were extracted and western blotted with antibodies against MAPKs (p-ERK, p-p38, and p-JNK) 30 min after UVB irradiation (5 mJ/cm^2); α-tubulin was used as a loading control. The results shown are representative of three independent experiments with similar results.

suppression of UVB-induced ROS generation was observed (Figure 4C). This suggests that ROS generation occurs through a BLT2-dependent pathway in response to low-dose UVB irradiation. In agreement with previous reports, we observed that pretreatment with diphenylene iodonium (DPI), an inhibitor of flavoenzymes such as NADPH oxidase, attenuates UVB-induced MMP-1 expression (Figure 4D). When taken together, these results suggest that the BLT2-ROS cascade mediates UVB-induced MMP-1 expression.

ERK phosphorylation lies downstream of the BLT2-ROS cascade in UVB signaling of MMP-1 in HaCaT cells

Extracellular-regulated kinase (ERK) is stimulated by elevated ROS and activates downstream signaling involving AP-1, which results in MMP expression (Whitmarsh and Davis, 1996; Shin *et al*., 2008; Shim *et al*., 2009). ERK stimulation may therefore lie downstream of the BLT2-ROS cascade to mediate MMP-1 expression in UVB-irradiated HaCaT cells. To test this hypothesis, we determined whether ERK phosphorylation is affected by pretreatment with the antioxidant DPI or by BLT2 knockdown with siBLT2. In agreement with previous observations reporting that mitogen-activated protein kinases (MAPKs), including ERK, play roles in UV-induced MMP-1 expression (Brenneisen *et al*., 2002; Wertz *et al*., 2004; Kim *et al*., 2005; Shin *et al*., 2008; Shim *et al*., 2009; Yang *et al*., 2009), ERK phosphorylation was greatly enhanced by low-dose UVB irradiation in HaCaT cells (Figure 5A). Additionally, JNK and p38 kinase levels were also stimulated by UVB irradiation, although the levels of phosphorylation were not as great as those of ERK. Pretreatment of the cells with PD98059 (an ERK inhibitor), SB203580 (a p38 kinase inhibitor), or SP600125 (a JNK inhibitor) clearly diminished the upregulation of MMP-1 in UVB-irradiated HaCaT cells (Figure 5B), suggesting that MAPK stimulation plays a role in the upregulation of MMP-1 in response to low-dose UVB irradiation. Next, to assess if ERK (or other MAPKs) stimulation lies downstream of BLT2 in UVB-irradiated cells, we tested the effect of BLT2 knockdown on UVB-induced MAPK stimulation. As shown in Figure 5C, BLT2 knockdown with siBLT2 clearly reduced ERK phosphorylation without affecting the phosphorylation of other MAPKs (i.e., JNK or p38 kinase). To further demonstrate the role of BLT2 in mediating UVB signaling of ERK phosphorylation, we examined whether BLT2 overexpression is sufficient for inducing ERK phosphorylation. We clearly observed greatly enhanced ERK phosphorylation by BLT2 overexpression alone (lane 2) compared to the control (Figure 5D). Additionally, further enhanced ERK phosphorylation was detected with UVB irradiation (Figure 5D). Taken together, these results suggest that ERK lies downstream of the BLT2-ROS cascade in the low-dose UVB-induced signaling pathway in HaCaT cells.

Discussion

In the present study, we demonstrated that a

BLT2-linked pathway is associated with low-dose UVB-induced MMP-1 expression in keratinocytes. This conclusion is based on the following observations: (1) BLT2 blockage using a specific antagonist or siRNA reduces low-dose UVB irradiation-induced MMP-1 expression; (2) BLT2 overexpression in HaCaT cells is sufficient to cause significant up-regulation of MMP-1 expression; (3) levels of both BLT2 ligands (LTB_4 and 12(S)-HETE) and the synthesizing enzymes for those ligands (5-LO and 12-LO, respectively) are considerably increased in response to UVB irradiation in HaCaT cells; and (4) blockage of the synthesis of BLT2 ligands with MK886 (an inhibitor of 5-LO) or Baicalein (an inhibitor of 12-LO) remarkably diminished UVB-induced MMP-1 expression. However the addition of LTB_4 or 12(S)-HETE to inhibitor-treated cells restored UVB-induced MMP-1 expression.

Additionally, we provide evidence suggesting that BLT2-mediated MMP-1 upregulation occurs through a signaling pathway dependent on ROS production and the subsequent stimulation of ERK. Consistent with our observations, ROS are known to mediate low-dose UVB-induced MMP expression (Brenneisen *et al*., 2002; Vayalil *et al*., 2004; Wertz *et al*., 2004). In particular, the importance of ROS in photo-aging is underlined by extensive evidence that exposure to antioxidants decreases UVB-induced MMP expression in dermal skin cells (Kang *et al*., 2003; Vayalil *et al*., 2004; Wertz *et al*., 2004; Zaid *et al*., 2007). However, neither the source of ROS generated in response to low-dose UVB irradiation nor the pathway by which they are generated in the skin has been clearly understood. Among the various sources of ROS, NADPH oxidase (Nox) is a major regulator of MMP-1 expression (Shin *et al*., 2008). Also, we recently reported that the BLT2 cascade mediates ROS generation through NADPH oxidase 1 (Nox1) (Ryu *et al*., 2010). We previously observed that BLT2 overexpression induces enhanced Nox1 mRNA expression and ROS generation in HaCaT cells (Ryu *et al*., 2010). Based on these observations, we suspect that Nox, possibly Nox1, is associated with BLT2-mediated ROS generation in response to low-dose UVB in HaCaT cells. In support of this idea, we observed that UVB-induced MMP-1 expression was significantly suppressed by pretreatment with DPI (Figure 4D). Nonetheless, the exact mechanism by which BLT2 mediates ROS generation in response to low-dose UVB irradiation requires further examination.

Moreover, in the present study, our results suggest that ERK stimulation lies downstream of the BLT2-ROS cascade (Figure 5) that mediates MMP-1 expression in UVB-irradiated HaCaT cells. In fact, our previous report showed that the BLT2-linked pathway regulates the high-dose UVB-induced sunburn response through signaling *via* the BLT2-Nox1-p38/JNK pathway (Ryu *et al*., 2010). Thus, although sunburn and photo-aging responses appear to share a common signaling mechanism, the downstream MAPKs involved in each response may be different. Whereas the sunburn response (induced by high-dose UVB) is likely to mainly be mediated through a pathway dependent on p38 kinase/JNK (Ryu *et al*., 2010), MMP-1 upregulation (and thus the photo-aging response triggered by low-dose UVB) is largely dependent on ERK. In support of the suggested role of ERK in photo-aging, but not sunburn apoptosis, ERK activation was shown to act as a survival signal in keratinocytes (He *et al*., 2004; Lee *et al*., 2005). Under our experimental conditions, the transient overexpression of BLT2 in HaCaT cells leads to remarkably enhanced ERK phosphorylation and MMP-1 upregulation (Figure 5D and Figure 2D), which again supports the close association of BLT2 with ERK stimulation. Similarly, our previous reports also show that ERK is activated by the BLT2 ligand (Woo *et al*., 2002, 2003). Nonetheless, we cannot exclude the possible contributory roles of p38 kinase or JNK in BLT2-mediated MMP-1 upregulation signaling. Although we suspect that ERK largely mediates the low-dose UVB-BLT2 pathway to upregulate MMP-1, other MAP kinases (e.g., p38 kinase and JNK) may play roles in the pathway, albeit to a much lesser extent.

MMP-1 is transcriptionally regulated by NF-κB and AP-1, which are activated by MAPKs (Fisher *et al*., 1996; Kim *et al*., 2005) or ROS (Garmyn and Degreef, 1997; Saliou *et al*., 1999). Consistent with this finding, we observed that pretreatment with the BLT2 antagonist LY255283 suppresses NF-κB/AP-1 transcriptional activity, whereas the BLT1 antagonist had no effect (data not shown). Thus, we speculate that NF-κB/AP-1 activation may lie downstream of the BLT2-linked pathway in UVB-induced MMP-1 upregulation in HaCaT cells. However, the detailed mechanism by which NF-κB/AP-1 activation contributes to the low-dose UVB-BLT2-linked pathway of MMP-1 upregulation must be further elucidated.

In summary, we demonstrated that BLT2 mediates low-dose UVB irradiation-induced MMP-1 expression in HaCaT cells. Additionally, we demonstrated that BLT2 mediates MMP-1 upregulation through a signaling pathway dependent on ROS production and the subsequent stimulation of ERK in UVB-irradiated HaCaT cells. Based on our findings, we propose that the BLT2-ROS-ERK-

linked pathway may potentially contribute to photo-aging and that this pathway could be an effective target for attenuating photo-aging in skin.

Methods

Cell culture and chemicals

The immortalized human skin keratinocyte HaCaT cell line was maintained in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS) in a 37°C, 5% CO_2 incubator. The cells were passaged every 3-4 days when they reached 80-90% confluency. All experiments were carried out in 60 mm dishes, and cells were grown to a confluent monolayer. The BLT2 antagonist LY255283, LTB_4 and 12(S)-HETE were purchased from the Cayman Chemical Company (Ann Arbor, MI). The BLT1 antagonist U75302 was from BioMol (Plymouth Meeting, PA). MK886, baicalein, and diphenyleneiodonium (DPI) were obtained from Calbiochem (La Jolla, CA). The monoclonal antibodies against MMP-1 were obtained from Calbiochem (La Jolla, CA). FBS and DMEM were from Gibco (Grand Island, NY). The polyclonal antibodies against MAP kinases (ERK, JNK, and p38 kinase) were obtained from Cell Signaling Technology (Danvers, MA). Anti-mouse and anti-rabbit secondary antibody horseradish peroxidase conjugates were purchased from Amersham Biosciences (Piscataway, NJ). The MAPK inhibitors PD98059, SB203580 and SP600125 were obtained from Calbiochem (San Diego, CA). All other chemicals were from standard sources and were of molecular biology grade or higher.

UVB irradiation

Cells were starved in serum-free DMEM for 24 h. Before UVB irradiation, the medium was removed, the cells were washed with warm PBS, and 200 μl of PBS was pipetted onto each dish to keep the cells wet. The cells were then irradiated with a UV crosslinker (Upland, CA), which incorporates 5 × 8 W tubes that emit most of their energy within the UVB range with an emission peak at 302 nm. The UVB dose was accurately calculated with a UVB meter. After irradiation, fresh serum-free DMEM was added to each dish.

Cell viability

Cell viability was assessed by the trypan blue exclusion assay 24 h after UVB irradiation. Whole cells were collected from the medium (detached cells) and culture dish (attached cells). A 10 μl aliquot of the cells was then suspended in DMEM, and 10 μl of 0.14% (w/v) trypan blue solution was added. Cells were counted using a hemocytometer under a light microscope. Cell viability is expressed as a percentage of the total cell population.

Semiquantitative RT-PCR for BLT1 and BLT2

Total cellular RNA was extracted from cells using an Easy Blue RNA Extraction kit (Intron Biotechnology, Seongnam, Korea), dissolved in diethylpyrocarbonate-treated water, and quantified by UV scanning. The RNA was reverse transcribed using Moloney murine leukemia virus reverse transcriptase (Invitrogen, Carlsbad, CA); BLT1, BLT2, and glyceraldehyde 3-phosphate (GAPDH) transcripts were amplified using a PCR PreMix kit (Intron Biotechnology, Seongnam, Korea). cDNA from 2 μg of total RNA was used for each PCR reaction. The primers for human BLT1 were 5'-TATGTCTGCGGAGTCAGCATGTACGC-3' (forward) and 5'-CCTGTAGCCGACGCCCTATGTCCG-3' (reverse). The PCR protocol for human BLT1 involved 30 cycles of denaturation at 94°C for 30 s, annealing at 67°C for 30 s, and elongation at 72°C for 30 s, followed by an extension at 72°C for 10 min. The primers for human BLT2 were 5'-TCTCATCGGGCATCACAGGT-3' (forward) and 5'-CCAAGCTCCACACCACGAAG-3' (reverse). The PCR protocol involved 35 cycles of denaturation at 94°C for 30 s, annealing at 65°C for 30 s, and elongation at 72°C for 30 s, followed by extension at 72°C for 10 min. The primers for GAPDH were 5'-CTGCACCACCAACTGCTTAGC-3' (forward) and 5'-CTTCACCACCTTCTTGATGTC-3' (reverse). The PCR protocol involved 22 cycles of denaturation at 94°C for 30 s, annealing at 58°C for 30 s, and elongation at 72°C for 30 s, followed by extension at 72°C for 10 min. The amplified PCR products were subjected to electrophoresis on 1.5% agarose gels, after which bands were visualized by ethidium bromide staining and photographed with a GelDoc system (BioRad, Hercules, CA). The specificity of all primers was confirmed by sequencing the PCR products. RNA extraction products were tested in control reverse transcription reactions and found to be free of DNA contamination.

Western blotting

To determine the levels of MMP-1 in culture, equal aliquots volume of conditioned culture medium, which were collected 48 h after UVB irradiation (5 mJ/cm^2) were separated by 10% SDS-PAGE and transferred to Hybond-P membranes (Amersham, NJ). The membranes were blocked and incubated with the primary antibody (MMP-1) and then with the horseradish peroxidase (HRP)-conjugated secondary antibody prior to development using an ECL kit (Amersham, NJ). The hybridized immunoblots were analyzed by autoradiography. Equal aliquots of whole cell lysates using the same volumes of RIPA lysis buffer were subjected to western blotting to measure α-tubulin as a loading control for MMP-1 PAGE. To assess MAPK phosphorylation, proteins were extracted using RIPA lysis buffer containing protease inhibitors 30 min after UVB irradiation.

RNAi of BLT2

The siBLT2 sequence (5'-CCACGCAGTCAACCTTCTG-3') was based on human BLT2 cDNA sequences (Hennig *et al*., 2008). HaCaT cells were transfected with scrambled (50 μM) or BLT2 specific siRNA (50 μM) using the Oligofectamine transfection reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions for RNA experiments. The mRNA levels of each gene were analyzed after 24 h by RT-PCR to evaluate interference.

Measurement of LTB_4 and 12-(S) HETE by ELISA

After UVB irradiation (5 mJ/cm^2), 500 μl aliquots of medium were removed at the indicated times (0, 10, 20, 30, and 60 min) and centrifuged at 12,000 rpm for 15 min at 4°C. The supernatants were freeze-dried overnight and reconstituted with the assay buffer supplied with the LTB_4 or 12(S)-HETE ELISA kits (Assay Designs, Ann Arbor, MI). LTB_4 and 12(S)-HETE were measured according to the manufacturers' instructions.

Measurement of ROS

HaCaT cells were starved in serum-free DMEM for 24 h before the intracellular ROS levels were measured. Cells were treated with 1 μM U75302 or 10 μM LY255283 for 1 h prior to UVB irradiation. After UVB irradiation for the indicated times, the levels of ROS were measured by FACS using Cell Quest Pro software (BD Bioscience, San Diego, CA). Before the measurement of intracellular H_2O_2, the cells were incubated for 20 min in the dark at 37°C with 20 μM 2',7'-dichlorofluorescein diacetate (Molecular Probes, Eugene, OR), an H_2O_2-sensitive fluorophore, as previously described (Woo *et al*., 2002).

MMP-1 promoter activity

HaCaT cells were transfected with 5 μg of human MMP-1 luciferase reporter construct using the Lipofectamine transfection reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. The MMP-1 luciferase reporter construct was kindly provided by Dr. Jin-Ho Chung (College of Medicine, Seoul National University; Kim *et al*., 2009). To monitor variations in cell number and transfection efficiency, HaCaT cells were cotransfected with 1 μg of pSV40-β-galactosidase, a eukaryotic expression vector containing the Escherichia coli β-galactosidase (lacZ) structural gene under control of the SV40 promoter. At 24 h after transfection, the cells were starved for 24 h in serum-free DMEM prior to UVB irradiation. Luciferase activity was measured using a Junior luminometer (Berthold, Germany) at 24 h after UVB irradiation (5 mJ/cm^2). The relative fold increase in luciferase activity was calculated as previously described (Woo *et al*., 2005).

Statistical analysis

All experiments were performed at least twice with similar results, and representative results are shown. The results are presented as means ± S.D. Analyses were performed with Student's *t*-test using SigmaPlot software (St. Louis, MO). Values of $P < 0.05$ were considered significant. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$.

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