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Modulation of the expression of membrane-bound CD54 (mCD54) and soluble form of CD54 (sCD54) in endothelial cells by glucosyl transferase inhibitor: possible role of ceramide for the shedding of mCD54☆

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Abstract

1-Phenyl-2-decanoylamino-3-morpholino-1-propanol (PDMP) is a synthetic inhibitor toward glucosyl transferase. Here, we showed the functional role of sphingolipids on CD54 expression of endothelial cells (ECs) by the use of PDMP. CD54 mRNA expression in human umbilical vein endothelial cells (HUVECs) was not changed by PDMP; however, PDMP treatment significantly enhanced the expression of membrane-bound CD54 (mCD54) on HUVECs. In contrast, the amount of soluble form of CD54 (sCD54) in the culture supernatants of HUVECs was diminished by PDMP. Similar results were obtained when HUVECs were incubated with metalloproteinase inhibitor, KB-R8301, or in the presence of C2-ceramide. The above effect of PDMP, KB-R8301, and C2-ceramide in HUVECs was commonly found in unstimulated, TNF-α-stimulated, and IL-1β-stimulated HUVECs. These data provide the possibility that the shedding of mCD54 into sCD54 by metalloproteinase-like enzyme is inhibited by PDMP, in which PDMP-induced accumulation of ceramide may act as a second messenger.

Vascular endothelial cells (ECs), lining the blood vessels, are now recognized as playing a pivotal role in a variety of human diseases, demonstrated by the fact that ECs present antigen to other immune cells, produce various kinds of cytokines, and recruit leukocytes into an inflammatory locus [1–4]. A number of humoral and cellular factors modulate endothelial cell functions, among which sphingolipids and their metabolic products are known to have second messenger functions in a variety of cellular signaling pathways [5–8].

Experimental studies using inhibitors toward sphingolipid biosynthesis have revealed a functional significance of sphingolipids. 1-Phenyl-2-decanoylamino-3-morpholino-1-propanol (PDMP) is a inhibitor for glucosyl transferase; thus, deficiency of glucosylceramide (GlcCer) as well as the accumulation of ceramide are developed in PDMP-treated cells [9–12]. PDMP treatment has been found to modulate a kind of cellular function [9–13]; however, its effect in ECs has not been fully examined. CD54 is a major functional molecule expressed in ECs. Membrane-bound CD54 (mCD54) on the surface of ECs acts as a costimulating/adhesion molecule toward T cell adhesion and activation through binding with lymphocyte function associated antigen 1 (LFA-1) [3,4]. Soluble form of CD54 (sCD54), developed from mCD54 by shedding [14], is also identified, and the increment of sCD54 in the sera of patients with lymphoma, leukemia, hypertension, and hepatitis has been determined [15–17].

We examined in the present study whether PDMP treatment modulates CD54 expression of human

☆ *Abbreviations:* ECs, endothelial cells; HUVECs, human umbilical vein endothelial cells; mCD54, membrane-bound CD54; sCD54, soluble form of CD54; PDMP, 1-phenyl-2-decanoylamino-3-morpholino-1-propanol; GlcCer, glucosylceramide; TNF-α, tumor necrosis factor-α; IL-1β, interleukin 1β; MFI, mean fluorescence intensity.

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umbilical vein endothelial cells (HUVECs). PDMP did not affect CD54 mRNA expression of HUVECs; however, the increment of mCD54 expression together with the decrement of sCD54 production in HUVECs was induced by PDMP. The use of metalloproteinase inhibitor, KB-R8301, as well as the addition of C2-ceramide mimicked the action of PDMP for CD54 expression of HUVECs. These data provide a possibility that the accumulation of ceramide in HUVECs is developed by PDMP, which suppresses the shedding of mCD54 on HUVECs through inhibiting the metalloproteinase-like enzyme activity.

Materials and methods

Cells and reagents. HUVECs were purchased from BioWhittaker (Walkersville, MD), and cultured in endothelial cell basal medium-2 (EBM-2, BioWhittaker) containing 2% FCS, according to manufacturer's protocol. Glucosyl transferase inhibitor, PDMP, and cell permeable C2-ceramide were obtained from Sigma Chemical (St. Louis, MO). Phycoerythrin (PE)-conjugated monoclonal antibody (mAb) toward human anti-CD54 (mouse IgG1), PE-conjugated control mouse IgG1, and sCD54 ELISA kit (sandwich ELISA kit) were purchased from MBL (Nagoya, Japan). Recombinant human tumor necrosis factor-α (rTNF-α) was purchased from R&D Systems (Minneapolis, MN) and human interleukin 1β was obtained from Otsuka Pharmaceutical (Osaka, Japan).

Detection of mCD54, sCD54, and CD54 mRNA in HUVECs. HUVECs were incubated in EBM-2 containing 2% FCS with or without varying concentrations of PDMP in the presence or absence of TNF-α (200 U/ml)/IL-1β (20 U/ml) for 24 h. After incubation, mCD54 expression on HUVECs and sCD54 accumulation in the culture supernatants were examined. mCD54 expression of HUVECs was studied by a flow cytometric analysis (Epics XL, Beckman Coulter, Hialeah, FL, mouse IgG1 was used as a negative control) and sCD54 protein concentration in the supernatants was examined according to manufacturer's protocol. The intensity of mCD54 expression on HUVECs was expressed as mean fluorescence intensity (MFI) in $mCD54^+$ cells determined by flow cytometer.

CD54 mRNA expression in HUVECs was investigated by RT-PCR analysis. In brief, total RNA from HUVECs, either unstimulated or stimulated with TNF-α/IL-1β, was extracted using TRIZOL (Life Technologies, Rockville, MD) and first-stranded cDNA was synthesized from total RNA by the use of ReveTra Ace (TOYOBO, Osaka, Japan). For PCR analysis, cDNA was amplified in 20 μl *Taq* polymerase buffer containing 1 U *Taq* DNA polymerase (TaKaRa Ex Taq: Takara Bionedicals, Tokyo, Japan), 1 μM of each primer, and 0.2 mM of each dNTP. A thermal cycle of 94 °C (1 min)–55 °C (1 min)–72 °C (45 s) was performed using a program of 30 cycles for CD54. β-Actin, internal control for PCR, was repeated for 30 cycles at 63 °C (30 s) of annealing. The amplified products were separated by electrophoresis on a 1.5% agarose gel. Primer pairs used for CD54; 5′-TATGGCAACGACTCCT TCT-3′ (sense) and 5′-CATTCAGCGTCACCTTGG-3′ (antisense), and for β-actin; 5′-TACATGGCTGGGGTGTTGAA-3′ (sense) and 5′-AAGAGAGGCATCCTCACCCT-3′ (antisense). Predicted size of each fragment is 238 and 218 bp, respectively. We also quantified the expression density of CD54 mRNA by the use of software NIH Image (1.61) by calculating the expression ratio of CD54 mRNA toward β-actin mRNA.

In some experiments, HUVECs were cultured in the presence of C2-ceramide (Sigma) or metalloproteinase inhibitor, KB-R8301 (Organon, Osaka, Japan), and the expression of mCD54 as well as the accumulation of sCD54 were examined.

Statistical analysis. Statistical analyses were performed using the Student's t test. p values < 0.05 were selected as the level of significance.

Results

Increment of mCD54 expression of HUVECs induced by PDMP

We initially examined the effect of PDMP on mCD54 expression of HUVECs. As shown in Fig. 1A, a dose-dependent increment of mCD54 on HUVECs was induced by PDMP. Augmentation of mCD54 expression on HUVECs by PDMP was also demonstrated in both TNF-α- and IL-1β-stimulated HUVECs (Fig. 1B). Regulatory role of PDMP on CD54 mRNA expression was also examined. As suspected, rapid CD54 mRNA expression in HUVECs was induced by TNF-α or IL-1β; however, PDMP did not change CD54 mRNA expression in unstimulated, TNF-α- or IL-1β-stimulated HUVECs (Fig. 2).

Modulation of shedding of mCD54 into sCD54 by PDMP

As shown in Fig. 3, although a little amount of sCD54 was found in the culture supernatants from untreated HUVECs, a significant accumulation of sCD54 was determined in the culture supernatants from TNF-α- or IL-1β-stimulated HUVECs. In contrast to PDMP-mediated increment for mCD54 expression, PDMP treatment clearly down-regulated the accumulation of sCD54 from unstimulated, TNF-α-, and IL-1β-stimulated HUVECs (Fig. 3).

We next examined the effect of metalloproteinase inhibitor, KB-R8301, on CD54 expression of HUVECs. As shown in Table 1, the similar findings regarding mCD54 expression as well as sCD54 accumulation were demonstrated in KB-R8301-treated HUVECs.

Ceramide is a sphingolipid accumulated in PDMP-treated cells; thus, we finally studied whether exogenous addition of ceramide mimicked the action of PDMP. Increment of mCD54 expression in unstimulated, TNF-α- or IL-1β-stimulated HUVECs was found by C2-ceramide (Fig. 4), and further, the amount of sCD54 determined in the supernatants was clearly decreased by C2-ceramide (Fig. 5). Apoptotic cell death of HUVECs, examined by the presence of hypodiploid DNA^+ cells and the disruption of mitochondrial transmemebrane potential, was not found throughout the experiments (data not shown).

Discussion

We tried to determine in the present study whether CD54 expression of ECs is regulated by sphingolipids by

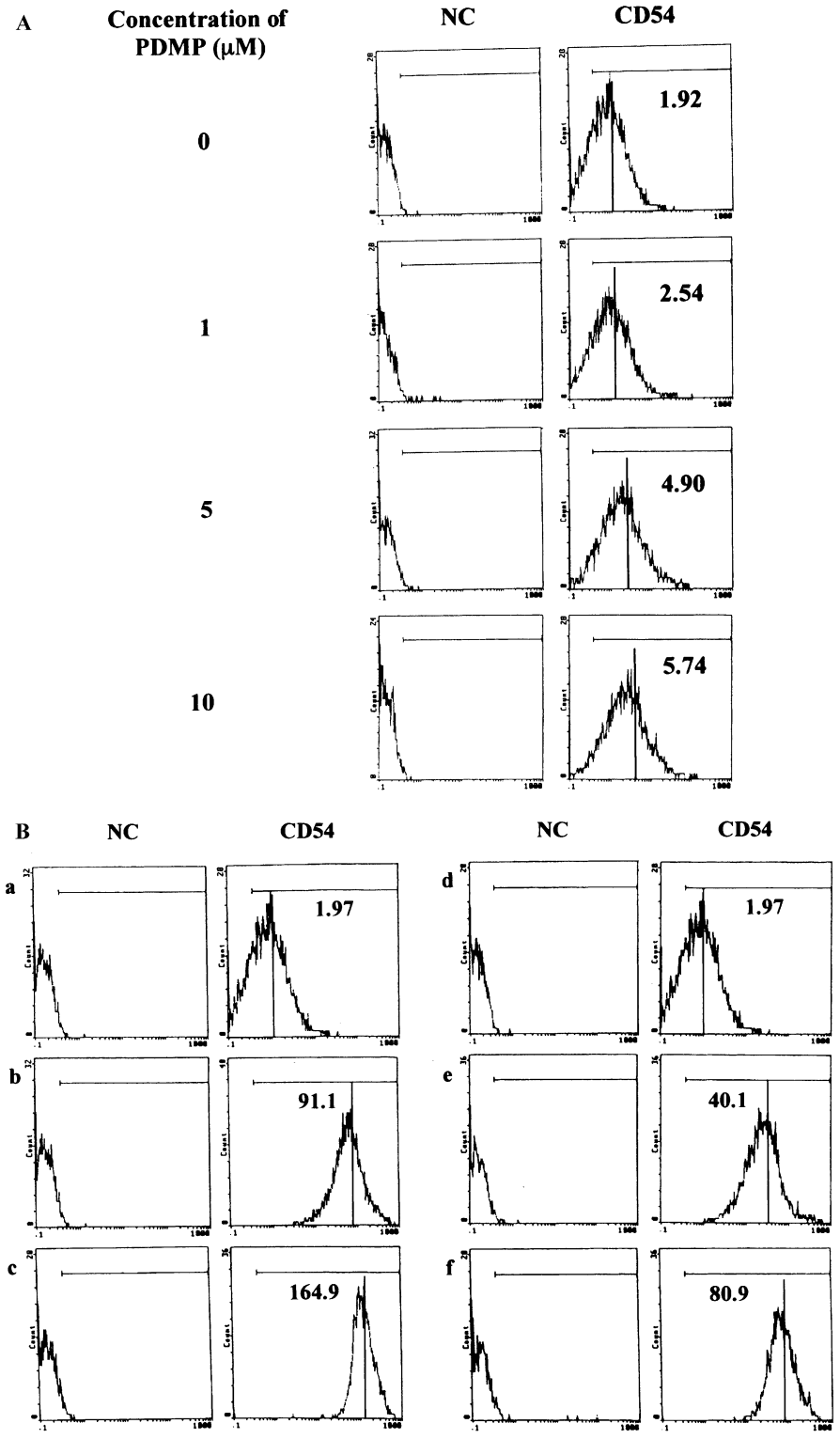

Fig. 1. Augmentation of mCD54 expression on HUVECs by PDMP. (A) HUVECs were cultured in the presence of varying concentrations of PDMP for 24 h. After cultivation, mCD54 expression on HUVECs was examined as described in the text. (B) HUVECs were cultured in the presence of 10 μM PDMP in the presence of TNF-α or IL-1β for 24 h. After cultivation, mCD54 expression on HUVECs was examined as described in the text. Note that mCD54 expression of HUVECs was dose-dependently increased by PDMP (A), and PDMP-mediated increment of mCD54 expression was also found in both TNF-α- and IL-1β-stimulated HUVECs (B). In (B), a and d, unstimulated HUVECs; b, TNF-α-stimulated HUVECs; c, TNF-α-stimulated HUVECs with 10 μM PDMP; e, IL-1β-stimulated HUVECs; f, IL-1β-stimulated HUVECs with 10 μM PDMP. Results shown are representative experiment from six performed. NC: negative control staining (mouse IgG1), CD54: stained with anti-CD54 mAb. The number appeared in this figure is an MFI of $CD54^{+}$ cells.

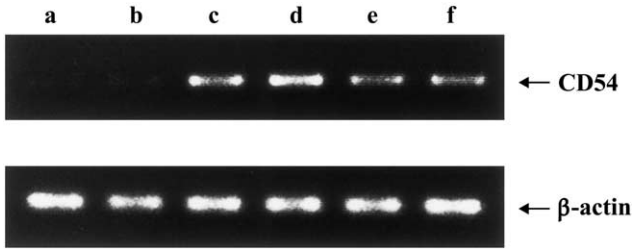

Fig. 2. RT-PCR analysis of CD54 mRNA expression in HUVECs. HUVECs were cultured in the presence or absence of 10 μM PDMP for 3 h with or without TNF-α/IL-1β. After cultivation, CD54 mRNA expression was examined as described in the text. a, Unstimulated HUVECs; b, HUVECs cultured with 10 μM PDMP; c, TNF-α-stimulated HUVECs; d, TNF-α-stimulated HUVECs with 10 μM PDMP; e, IL-1β-stimulated HUVECs; f, IL-1β-stimulated HUVECs with 10 μM PDMP. CD54 mRNA expression in HUVECs was clearly increased by TNF-α or IL-1β. CD54 mRNA expression of HUVECs, either in unstimulated or TNF-α-/IL-1β-stimulated, was not changed by PDMP. Results shown are representative experiment from four performed.

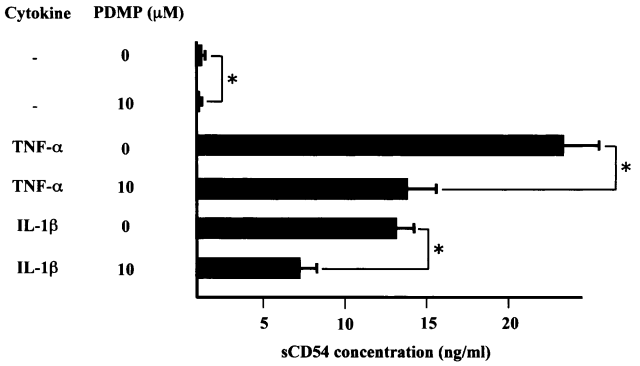

Fig. 3. PDMP-mediated reduction of sCD54 accumulation in the culture supernatants from HUVECs. HUVECs were cultured in the presence or absence of 10 μM PDMP for 24 h with or without TNF-α/IL-1β. After cultivation, protein concentration of sCD54 in the culture supernatants was examined as described in the text. sCD54 accumulation was significantly increased by TNF-α or IL-1β. sCD54 accumulation in the supernatants from unstimulated, TNF-α- and IL-1β-stimulated HUVECs was clearly suppressed by PDMP. Results shown are means ± SD of sCD54 protein concentrations from five individual experiments. *, $p < 0.01$, vs absence of PDMP.

the use of glucosyl transferase inhibitor, PDMP. PDMP treatment of HUVECs augmented mCD54 expression (Fig. 1) without changing CD54 mRNA expression (Fig. 2). PDMP-mediated increment in mCD54 expression appears to be generally demonstrated, since the phenomenon was found in both unstimulated and cytokine-stimulated HUVECs. Protein expression of CD54 is sometimes not associated with CD54 mRNA expression [18], which suggests the importance of posttranscriptional and/or posttranslational regulation in CD54 expression. mCD54 is proved to be shed into a soluble form [14], and interestingly, the present study showed that PDMP treatment augmented mCD54 expression on HUVECs with decrement of sCD54 accumulation.

Table 1
Effects of KB-R8301 on CD54 expression from HUVEC

Cytokine	KB-R8301 (μM)	MFI of mCD54	sCD54 concentration (ng/ml)
–	0	$1.8 \pm 0.1^*$	$0.65 \pm 0.05^*$
–	10	$43 \pm 0.2^*$	$0.42 \pm 0.04^*$
TNF-α	0	$99.5 \pm 5.2^*$	$24.7 \pm 1.7^*$
TNF-α	10	$158.4 \pm 7.1^*$	$16.5 \pm 1.2^*$
IL-1β	0	$45.5 \pm 2.1^*$	$13.8 \pm 0.8^*$
IL-1β	10	$74.7 \pm 3.1^*$	$8.4 \pm 0.4^*$

HUVECs were cultured in the presence or absence of 10 μM KB-R8301 for 24 h with or without TNF-α/IL-1β. After incubation, the expression of mCD54 as well as sCD54 accumulation was examined as described in the text. Note that the increment of mCD54 expression together with the reduction of sCD54 accumulation was induced by KB-R8301. Results shown are means ± SD from five individual experiments.

* $p < 0.01$, vs absence of KB-R8301.

These data suggest that some posttranslational mechanisms, including the shedding process of mCD54 into sCD54, play an important role in PDMP-mediated regulation of CD54 expression in HUVECs. Although the protease to cleave mCD54 remains to be determined, the present study showed that the use of metalloproteinase inhibitor, KB-R8301, mimicked the action of PDMP, suggesting that PDMP suppresses the activity of metalloproteinase-like enzyme, and thus, increases mCD54 expression of HUVECs, which is responsible for the decrement of sCD54. Metalloproteinase-like enzyme-mediated release of membrane-bound molecule into a soluble one has been demonstrated in other cases, such as Fas ligand, TNF-α and receptor activator of NF-κB ligand (RANKL, [19–21]).

Ceramide is a sphingolipid accumulated in PDMP-treated cells [9,10,12]; thus, the effect of ceramide for CD54 expression of HUVECs was investigated. The similar biologic effect was found in C2-ceramide-treated HUVECs as PDMP-treated HUVECs. Although we did not measure ceramide production from HUVECs in the present study, previous studies identified the increase of endogenous ceramide production by TNF-α and IL-1β [22,23]. Thus, a certain amount of ceramide in HUVECs, especially in TNF-α- and IL-1β-stimulated HUVECs, may be endogenously produced; however, the present data obtained by adding exogenous C2-ceramide imply the importance of ceramide accumulation to modulate CD54 expression of HUVECs. Regulatory role of ceramide acting on kinases activity in association with the activation of caspases has been identified [24,25]. Taken together, it is possible to speculate that ceramide functions as a second messenger during PDMP treatment with HUVECs and suppresses a candidate protease, which is responsible for the shedding of mCD54. Previous study showed that the deficiency of GlcCer was also developed by PDMP treatment [11], which could also involve in CD54 regulation of

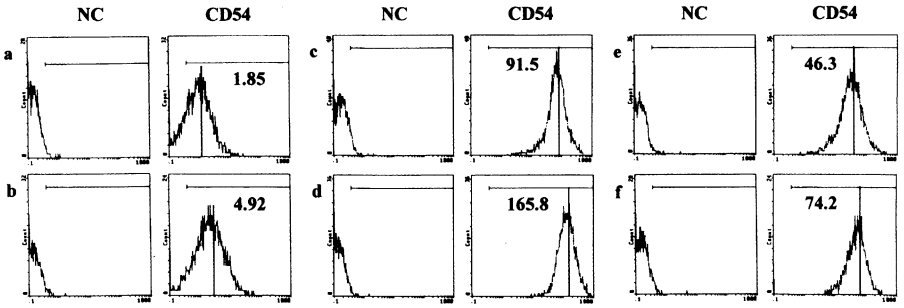

Fig. 4. Increment of mCD54 expression on HUVECs by C2-ceramide. HUVECs were cultured in the presence or absence of 10 μM C2-ceramide for 24 h with or without TNF-α/IL-1β. After cultivation, mCD54 expression was examined as described in the text. a, unstimulated HUVECs; b, HUVECs cultured with 10 μM C2-ceramide; c, TNF-α-stimulated HUVECs; d, TNF-α-stimulated HUVECs with 10 μM C2-ceramide; e, IL-1β-stimulated HUVECs; f, IL-1β-stimulated HUVECs with 10 μM C2-ceramide. Note that the increment of mCD54 expression by C2-ceramide was demonstrated both in unstimulated and TNF-α-/IL-1β-stimulated HUVECs. NC: negative control staining (mouse IgG1), CD54: stained with anti-CD54 mAb. The number appearing in this figure is an MFI of $CD54^+$ cells. Results shown are representative experiment from four performed.

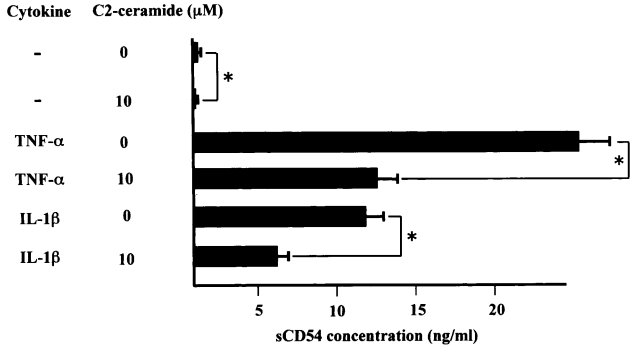

Fig. 5. C2-ceramide-mediated increment of sCD54 accumulation in the culture supernatants from HUVECs. HUVECs were cultured in the presence or absence of 10 μM C2-ceramide for 24 h with or without TNF-α/IL-1β. After cultivation, protein concentration of sCD54 in the culture supernatants was examined as described in the text. sCD54 accumulation was significantly increased by TNF-α or IL-1β. sCD54 accumulation in the supernatants from unstimulated, TNF-α- and IL-1β-stimulated HUVECs was markedly suppressed by C2-ceramide. Results shown are means ± SD of sCD54 protein concentrations from five individual experiments. *, $p < 0.01$, vs absence of C2-ceramide.

HUVECs by PDMP. Further studies should be necessary to find out essential sphingolipids involved in CD54 expression of HUVECs.

This is the first report demonstrating the regulation of CD54 expression in ECs by sphingolipids. Sphingolipids, including ceramide, are suggested to modulate the protease activity, thus, regulate CD54 expression at posttranslational level. Since sphingolipid biosynthesis is managed by both physiologic and pathologic stimuli including by cytokines, hormones, and viruses [23,26–28], our present data may provide a new insight into understanding the vascular pathophysiology.

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