Daunorubicin attenuates tumor necrosis factor-α-induced biosynthesis of plasminogen activator inhibitor-1 in human umbilical vein endothelial cells

Shinji Soeda, Kenji Iwata, Yoshiko Hosoda, Hiroshi Shimeno *

Department of Biochemistry, Faculty of Pharmaceutical Sciences, Fukuoka University, 8-19-1 Nanakuma, Jonan-ku, Fukuoka 814-0180, Japan

Received 4 October 2000; received in revised form 15 January 2001; accepted 16 January 2001

Abstract

The anthracycline antibiotic daunorubicin is reported to induce apoptosis in cells by triggering ceramide generation through de novo synthesis or sphingomyelin hydrolysis. Treatment of human umbilical vein endothelial cells (HUVEC) with daunorubicin markedly decreased the mRNA expression and protein release of plasminogen activator inhibitor-1 (PAI-1). This cellular event was accompanied by a significant increase in the total ceramide content in HUVEC. On the other hand, tumor necrosis factor (TNF)-α treatment of HUVEC led to an increase in both PAI-1 mRNA expression and protein release, and an enhancement of total ceramide content was also observed. The stimulating effect of TNF-α on PAI-1 synthesis was attenuated by the pretreatment of HUVEC with daunorubicin. Interestingly, the daunorubicin-induced increase in ceramide content was blocked by addition of the potent ceramide synthase inhibitor fumonisin B1, while the TNF-α-induced ceramide increase was not affected by this drug. Fumonisin B1 treatment restored the daunorubicin-induced decrease in PAI-1 release to approximately 70% of the control, but did not affect the TNF-α-induced increase in PAI-1 release. Thus, these data imply the possibility that the subcellular topology of ceramide production determines its lipid mediator function in the regulation of PAI-1 synthesis in HUVEC, because both TNF-α and daunorubicin could increase the ceramide levels. © 2001 Elsevier Science B.V. All rights reserved.

Keywords: Plasminogen activator inhibitor-1; Daunorubicin; Tumor necrosis factor-α; Ceramide; Vascular endothelial cell

1. Introduction

Plasminogen activator inhibitor-1 (PAI-1) is an important physiological regulator of fibrinolysis. PAI-1 rapidly inactivates tissue-type and urokinase-type plasminogen activators (PAs) and blocks the generation of the fibrinolytic enzyme plasmin. The expression of PAI-1 is enhanced in atherosclerotic plaques [1–3], and a linkage between an excess of circulating [4] or arterial [5] PAI-1 and the risk of intravascular thrombotic events has been reported.
Macrophages, which are central cell types in atherosclerosis, produce large amounts of cytokines such as interleukin 1 and tumor necrosis factor (TNF-α). TNF-α stimulates PAI-1 synthesis by various cell types in vitro and in vivo [6-10]. However, the TNF-α signaling events leading to PAI-1 production are not yet fully understood.

We have recently demonstrated that TNF-α-induced release of PAI-1 from human umbilical vein endothelial cells (HUVEC) is accompanied by an elevation of intracellular ceramide levels, and suggested an important role of the ceramide as the signaling molecule [9]. TNF-α may activate the sphingomyelin (SM) pathway located in the endosomal/lysosomal compartments [9] and/or in the plasma membrane [11], where the activated sphingomyelinase (SMase) hydrolyzes SM to generate ceramide. However, a recent report [12] suggests that the elevation of de novo synthesis of ceramide is involved in TNF-α signaling of cultured cerebral endothelial cells. De novo ceramide synthesis occurs in microsomes via activation of ceramide synthase (EC 2.3.1.24) [13]. The anthracycline antibiotic daunorubicin could activate this enzyme to induce apoptosis in P388 and U937 cells [14]. This finding has also been challenged by Jaffrézou et al. [15], where ceramide generation via SM hydrolysis underlies the drug-triggered apoptosis in HL-60 and U937 cells. The response of cells to daunorubicin may differ between various cell types and depend on the drug concentration used. Additionally, how long the cells are exposed to the drug may determine the ceramide generation mechanism.

In the present study, to examine the effect of daunorubicin on HUVEC, the cells were treated for 30 min with a high concentration (20 μM) of the drug, washed out, and incubated in the culture medium alone. These procedures may at least prevent the injurious actions of daunorubicin on HUVEC. We report that the incorporated daunorubicin suppresses the PAI-1 mRNA expression and protein synthesis in HUVEC after an increase in cellular ceramide levels. We further show that TNF-α also increases the ceramide levels in HUVEC but conversely enhances the biosynthesis of PAI-1. The TNF-α-induced increase in PAI-1 synthesis is attenuated by daunorubicin pretreatment. Fumonisin B₁ is a potent and specific inhibitor of ceramide synthase with no obvious effects on other enzymes of SM metabolism [16]. This agent could restore the daunorubicin-induced decrease in PAI-1, but did not affect the TNF-α-induced increase in PAI-1 release. Our present data suggest that daunorubicin mainly utilizes de novo synthesized ceramide for the down-regulation of PAI-1 gene expression in HUVEC. In contrast, TNF-α may trigger ceramide generation via SM hydrolysis to up-regulate PAI-1 synthesis.

2. Materials and methods

2.1. Materials

The following reagents were commercially obtained: daunorubicin, fumonisin B₁, N-palmitoyl-D-sphingosine (C₁₆-ceramide) from Sigma; and recombinant human TNF-α (1.0×10⁸ U/mg protein) from Boehringer Mannheim.

2.2. Cell culture and treatment

Cryo HUVEC and the culture medium EGM-2 were purchased from Sanko Junyaku, Tokyo, Japan. EGM-2 is composed of modified MCDB 131 medium and supplements that contain fetal bovine serum, epidermal growth factor, basic fibroblast growth factor, insulin-like growth factor, vascular endothelial growth factor, heparin, ascorbic acid, hydrocortisone, amphotericin B and gentamicin. The cells were grown in EGM-2 at 37°C in a humidified 5% CO₂ atmosphere. All experiments were performed with HUVEC at passages 3-5. For experiments, HUVEC were seeded in 6-well plates at a density of 2.5×10⁵ cells/cm² and allowed to reach confluence in EGM-2. After removal of the conditioned medium, the cell layer was rinsed twice with GIT medium (Daigo Eiyo, Osaka, Japan), which is serum-free but contains 3 mg/ml growth factors isolated from bovine serum (corresponding to the addition of 10% fetal bovine serum), 2 μg/ml insulin, 2 μg/ml transferrin, 122 ng/ml ethanalamine, amino acids, vitamins, and inorganic components. Prior to the treatment with TNF-α, HUVEC were pretreated at 37°C for 30 min in 3 ml of GIT medium supplemented with the indicated agent and rinsed with GIT medium. Incubation followed at 37°C for a definite
time in 3 ml of GIT medium. Daunorubicin and fumonisin B₁ were dissolved in ethanol. Control cells were treated with identical volumes of ethanol vehicle.

2.3. Assay of PAI-1 antigen

Antigen levels of PAI-1 in the conditioned media were determined with an enzyme-linked immunosorbent assay (ELISA) kit, Imulyse PAI-1 (Biopool, Umeå, Sweden), according to the manufacturer’s instructions. This assay detects the free form of PAI-1, but not the PA/PAI-1 complex.

2.4. Determination of total ceramide content

HUVEC were maintained in GIT medium at 37°C for a definite time, after pretreatment for 30 min with the indicated agent. The cells were detached, centrifuged, and washed with cold phosphate-buffered saline (PBS). The washed cells (1×10⁶) were resuspended in 220 μl of cold PBS. The cell suspension was mixed with 4 ml of chloroform/methanol (2:1, v/v) and extracted for 15 min. After the addition of 1 ml water, the sample was vortexed and centrifuged. The lower layer was collected and evaporated to dryness under a nitrogen stream. The amount of C₁₆-ceramide in the residue was quantitated by liquid chromatography/ion spray ionization mass spectrometry (LC/MS) according to the method described by Mano et al. [17] with minor modifications. Briefly, high-performance liquid chromatography (HPLC) was performed using a Gulliver 1500 series HPLC system (Jasco, Tokyo, Japan) equipped with a Develosil ODS HG-5 reversed-phase column (35×2.0 mm i.d., 5 μm, Nomura Chemical, Aichi, Japan). The mobile phases were as follows: A, 5 mM ammonium formate/methanol/tetrahydrofuran (5/2/3, v/v); and B, 5 mM ammonium formate/methanol/tetrahydrofuran (1/2/7, v/v) containing 0.01% formic acid. Elution was performed at a flow rate of 0.2 ml/min with the following modification: 70% mobile phase A–100% mobile phase B for 6.3 min in a linear gradient mode. LC/MS analyses were done with an electrospray ionization mass spectrometer (Finnigan-LCQ) and Navigator (Finnigan).

2.5. RNA extraction and RT-PCR analysis

Total RNA was isolated from either control or the indicated agent-treated HUVEC, using an RNasea mini kit (Qiagen). The synthesis of first strand cDNAs was performed with the Superscript system (Gibco BRL), according to the manufacturer’s instructions. Oligonucleotide primers were prepared as described by Hill et al. [18]. The PAI-1 forward 5’-CGGAGCACGGTCAAGCAAGTG-3’ and PAI-1 reverse 5’-GTTGAGGCGAGAGAGGRC-3’ primers were used to amplify a 400-bp fragment near the 3’ end of the PAI-1 cDNA. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) forward 5’-CATGCCAAATTTCACTGGCA-3’ and reverse 5’-TCTAGACCGAGGTAGTCCACC-3’ primers were used to amplify a 600-bp fragment. Polymerase chain reaction (PCR) amplification, using cDNA from the reverse transcriptase (RT) reaction, was performed with the PCR Reagent System (Gibco BRL) in a Program temp.control system (PC-701, Astec, Fukuoka, Japan). We selected a number of 25 cycles. PCR started for 2 min at 94°C followed by cycles consisting of: 60 s at 60°C, 60 s at 72°C, and 60 s at 94°C. Amplification was terminated after 7 min at 72°C. Products were visualized and photographed under UV radiation following 1% agarose gel electrophoresis.

3. Results

3.1. Effect of daunorubicin on PAI-1 expression in HUVEC

We first tested the effects of daunorubicin and fumonisin B₁ which are the activator [14,15] and inhibitor [16], respectively, of ceramide generation, on PAI-1 mRNA and protein levels in HUVEC. HUVEC were pretreated for 30 min with each agent, rinsed, and incubated in the conditioned medium alone. Daunorubicin dose-dependently decreased the PAI-1 antigen levels in media as early as 6 h after the treatment; the suppression lasted for at least 24 h (Fig. 1A). This was in contrast to the effect seen with TNF-α [9], where the PAI-1 release was enhanced in
a time- and concentration-dependent manner. Fumonisin B1 pretreatment did not affect the PAI-1 antigen levels (Fig. 1B). Fig. 2 shows that daunorubicin, but not fumonisin B1, reduced the PAI-1 mRNA levels slightly at 5 µM and clearly at 20 µM at 6 h (PAI-1 mRNA at 6 h appears to be wiped out, but, truly, a small amount of the mRNA is expressed at that time. An increase in cycles of PCR could visualize it). These daunorubicin effects are expected to have resulted from the increased intracellular ceramide levels following the activation of ceramide synthase [14] or SMase [15]. Therefore, we quantitated the C16-ceramide content in these cells by LC/MS analysis (Fig. 3). The pretreatment with 20 µM daunorubicin for 30 min and following incubation for 2 h in the conditioned medium alone increased the ceramide content to 2.8-fold of the control (61.5 ± 5.95 ng vs. 171 ± 8.57 ng, P < 0.001). Changes in the ceramide content were not detected with fu-
monisin B₁ treatment. Thus, the daunorubicin-induced suppression of PAI-1 expression in HUVEC may be closely related to primarily increased ceramide (increase in antigen levels, 6–24 h; in mRNA levels, 3–6 h; and in ceramide levels, 2 h). In contrast, fumonisin B₁ pretreatment had little or no effect on the ceramide levels and PAI-1 expression. One possible explanation for the lack of effect of fumonisin B₁ may be that HUVEC do not operate the ceramide synthase under these culture conditions without the activator, daunorubicin. However, it remains to be determined whether the daunorubicin-induced increase in ceramide content is due to activation of ceramide synthase [14] or of SM hydrolysis [15].

3.2. TNF-α-induced PAI-1 expression in HUVEC is attenuated by daunorubicin

We previously investigated the biochemical mechanism of the TNF-α-induced release of PAI-1 from HUVEC, and suggested that ceramide generation underlay the induction of PAI-1 release [9]. We next tested the effect of daunorubicin on the TNF-α-induced increase in PAI-1 synthesis. HUVEC were pretreated with 1, 5, or 20 μM daunorubicin for 30 min, rinsed, and exposed to a saturated concentration (500 U/ml) of TNF-α (Fig. 4A). After a 6-h incubation of HUVEC with TNF-α alone, the PAI-1 antigen levels in media rose from 166 ± 25.0 to 311 ± 21.3 ng/ml (1.9-fold increase, compared to the control). Pretreatment with daunorubicin caused a suppression of the TNF-α-induced increase in PAI-1 release: the concentration-dependent suppression lasted for at least 24 h. Fig. 4B shows the expression of PAI-1 and GAPDH mRNAs at 3 and 6 h. A 6-h exposure of HUVEC to 500 U/ml TNF-α caused an elevation of PAI-1 mRNA levels, compared to the control. However, the TNF-α-induced increase in PAI-1 mRNA levels was suppressed by daunorubicin pretreatment in a concentration-dependent manner (PAI-1 mRNA at 6 h appeared to be wiped out, but, truly, a small amount of the mRNA is expressed at that time. An increase in cycles of PCR could visualize it).

We next determined the effect of TNF-α on the total ceramide content in HUVEC (Fig. 5A). After a 2-h exposure of HUVEC to TNF-α (500 U/ml), the ceramide content was increased to 156% of the control (79.4 ± 7.68 ng vs. 123 ± 8.36 ng, P < 0.001). Pretreatment of HUVEC with 20 μM fumonisin B₁ did not affect the TNF-α-induced increase in ceramide content (119 ± 13.1 ng). On the other hand, fumonisin B₁ (20 μM) reduced the daunorubicin-induced increase in ceramide levels to near the control (96.0 ± 14.9 ng). Additionally, daunorubicin plus TNF-α treatment increased the total ceramide content, but there was no significant difference between the treatments with daunorubicin plus TNF-α and daunorubicin alone. Daunorubicin treatment might maximally elevate the ceramide levels in HUVEC.
These data strongly suggest that TNF-α-induced ceramide is not the ceramide that resulted from daunorubicin treatment. As shown in Fig. 5B, pretreatment with fumonisin B1 did not affect the TNF-α-induced release of PAI-1, but restored the daunorubicin-induced suppression of PAI-1 release to approximately 70% of the control. Therefore, these data suggest that TNF-α signals to mainly generate ceramide via SM hydrolysis and that, conversely, a large part of the ceramide generated by daunorubicin is de novo synthesized product. Furthermore, TNF-α- and daunorubicin-induced changes in PAI-1 biosynthesis respond well to the increase in ceramide generation, suggesting that, in both cases, the generated ceramides act as the up- and down-regulators of PAI-1, respectively.

4. Discussion

Daunorubicin has been proven to have therapeutic benefit in the treatment of a variety of neoplasias. However, its mechanism of anti-tumor action is not yet fully understood, although DNA is believed to be a primary target [19]. Recently, daunorubicin was shown to increase ceramide levels in cells following the induction of ceramide synthase [14] or of SM hydrolysis by SMase [15]. Furthermore, inhibition of ceramide synthase by the mycotoxin fumonisin B1 blocks apoptosis induced by daunorubicin [14]. Multiple experimental data support the notion that ceramide generated by SM hydrolysis plays an important role as the putative second messenger in regulating such diverse responses as cell cycle arrest, apoptosis, and cell senescence [20], although the concept has recently been challenged [21].

Here, we have shown that daunorubicin reduces the expression and biosynthesis of PAI-1 in HUVEC. PAI-1 mRNA decreased within 6 h in the cells, and this decrease was reflected in PAI-1 antigen levels up to at least 24 h. The early increase in total ceramide content (within 2 h) strongly suggests that ceramide plays an important role in the suppression of PAI-1 biosynthesis. Fumonisin B1 (20 μM) treatment restored the daunorubicin-induced decrease in PAI-1 antigen release to approximately 70% of the control. The maximal restoration decreased with decreasing concentrations of fumonisin B1. Treatment of HUVEC with 1 μM fumonisin B1 had no effect, but with 5 μM the agent restored the reduction of PAI-1 release to 50% of the control (data not shown). These results indicate that daunorubicin mainly induces the activation of ceramide synthase in HUVEC within
2 h and that the generated ceramide may act as the lipid messenger to reduce PAI synthesis and release. However, the partial restoration of PAI-1 release by fumonisin B₁ treatment suggests that ceramide is partly generated by SM hydrolysis, and how the ceramide regulates the PAI-1 synthesis remains obscure. Recently, Jaffrézou et al. [15] have shown that exposure of HL-60 cells to 1 μM daunorubicin for 4–10 min and 60–70 min concomitantly increases ceramide levels by neutral SMase, but not by ceramide synthase. The authors suggest an important role of the early SM-derived ceramide in daunorubicin-induced apoptosis. Here, we determined the ceramide levels in HUVEC at 2 h after the pretreatment procedures with 20 μM daunorubicin. The exposure of HUVEC for only 30 min to the high concentration of daunorubicin may have greatly influenced the neutral SMase present in plasma membranes. The time course of decrease in PAI-1 expression following the increase in ceramide levels seems to be convincing to explain the function of de novo synthesized ceramide as the down-regulator of PAI-1 synthesis.

TNF-α also increased the ceramide content in HUVEC within 2 h, but conversely enhanced the biosynthesis of PAI-1. Additionally, the elevation of ceramide at 2 h was not blocked by the ceramide synthase inhibitor fumonisin B₁. This result implies that the TNF-α-induced ceramide is mainly caused by SM breakdown, but not by de novo synthesis [12]. To date, seven different SMases have been found in mammalian cells, tissues, and biological fluids [20]. Previously, we observed a significant elevation of acidic lysosomal SMase activity in TNF-α-treated HUVEC [9]. However, the acidic SMase gene gives rise to both lysosomal SMase and secretory SMase, which is fully or partially dependent on Zn²⁺ for enzyme activity [22]. Segui et al. [23] have recently shown that TNF-α does not induce the generation of ceramide by acidic SMase during apoptosis in cells. At present, we have no data to bring forward to the apoptotic role of acidic SMase and its product, ceramide. However, TNF-α increases PAI-1 biosynthesis at least by increasing ceramide levels, which are not de novo synthesized but are a SM breakdown product. It is adequate to conclude that the increased ceramide is due to SM hydrolysis by the other SMase(s) or by the acid SMase gene product. TNF-α increased both PAI-1 mRNA and PAI-1 antigen levels within 3 and 6 h, respectively. These time course data strongly suggest that the ceramide generated at 2 h is closely related to the effect of TNF-α on PAI-1 expression, but there is at present no crucial evidence that ceramide is the signaling molecule to regulate PAI-1 synthesis. Taken together, our present findings suggest that, if ceramides induced by daunorubicin and TNF-α act as the signal messenger, the subcellular topology of ceramide production is important for determining the ceramide signaling to regulate PAI-1 expression in HUVEC.

Finally, pathological roles of elevated PAI-1 levels in coronary artery disease [1–5] and in a number of tumors [24–26] have been reported. An interesting role of PAI-1 on tumor cells has recently been suggested: the bad prognostic factor, PAI-1, released from tumor cells inhibits their apoptotic process [27]. The present data that daunorubicin could decrease PAI-1 biosynthesis in HUVEC suggest a possible role of this drug as a down-regulator of PAI-1 expression in tumors. We treated HUVEC with 20 μM daunorubicin but removed the drug from the cell culture after 30 min. The amount of daunorubicin incorporated into the cells may be near to the clinically achievable concentrations (≤5 μM). If the drug were left in the cell culture, 2 μM daunorubicin induces a similar decrease in PAI-1 release from HUVEC (data not shown). The suppressive effects of daunorubicin on PAI-1 synthesis may be an additional therapeutic benefit of this drug for cancers.

Acknowledgements

We gratefully acknowledge Mr. Hideyuki Yoshida, Faculty of Pharmaceutical Sciences, Fukuoka University for technical instruction in LC/MS analysis. This work was supported in part by a Grant-in-Aid for Scientific Research (C) from the Ministry of Education, Science, Sports and Culture of Japan and by funds from the Central Research Institute of Fukuoka University.

References


