Thrombosis Research 135 (2015) 1154–1159

Contents lists available at ScienceDirect

Thrombosis Research

journal homepage: www.elsevier.com/locate/thromres

Regular Article

Thalidomide and multiple myeloma serum synergistically induce a hemostatic imbalance in endothelial cells in vitro

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ARTICLE INFO

Article history:
Received 11 June 2014
Received in revised form 12 February 2015
Accepted 12 March 2015
Available online 18 March 2015

Keywords:
Deep vein thrombosis
Endothelium
Multiple myeloma
Serum
Thalidomide

ABSTRACT

Background: Thalidomide (Thal) treatment of patients with multiple myeloma (MM) is associated with vascular thrombosis, but the underlying mechanism is unknown.
Objectives: To evaluate the hypothesis that Thal, dexamethasone (Dex) and MM serum perturb the hemostatic balance on human umbilical vein endothelial cells (HUVECs).
Methods: Drugs with or without the serum of MM patients or healthy controls were incubated with HUVECs. Analyses of phosphatidylserine (PS), tissue factor (TF), endothelial protein C receptor (EPCR) and thrombomodulin (TM) were performed using flow cytometry. The production of thrombin and activated protein C (APC) were measured by chromogenic assay. The roles of IL-6 and TNFα in regulating these indicators were also investigated.
Results: We found that Thal or Dex alone could not increase TF and PS expression in HUVECs. However, when pretreated with MM serum, their expression was significantly increased by either Thal or Dex. Concurrent changes were also detected in thrombin generation. In contrast, Thal and Dex had a direct inhibitory effect on the expression of EPCR and TM, and this inhibitory effect was especially significant when MM serum was added. The generation of APC paralleled the expression of EPCR and TM. All of the above outcomes were reversed to a variable extent by anti-IL-6R and anti-TNFα antibodies.
Conclusions: These findings suggest Thal may act as a procoagulant by altering the balance between procoagulant and anticoagulant proteins on the surface of HUVECs, thereby contributing to thrombogenesis. MM serum plays a synergistic role in this process.

Introduction

Thalidomide (Thal) is an important therapeutic option for patients with multiple myeloma (MM) due to its powerful efficacy and low cost. However, its value is hindered by an increased incidence of venous thrombosis (VTE) [1].This side effect is particularly prominent when Thal is administered in combination with high-dose dexamethasone (Dex) [1]. Although many studies have been carried out in this field, the exact mechanism underlying this adverse effect remains unknown.

The endothelium plays a crucial role in maintaining the proper hemostatic balance.

After activation, various endothelial properties can be modulated to promote clot formation through the coordinated induction of procoagulant mechanisms and suppression of anticoagulant mechanisms [2]. Upon diagnosis or after Thal therapy, patients with MM exhibit pathologically enhanced von Willebrand factor (vWF), a marker of endothelial activation [3,4], which supports the hypothesis that endothelium activation plays an important role in thrombus formation in patients with MM. Unlike conventional cytotoxic chemotherapy, Thal suppresses tumor angiogenesis; moreover, it acts primarily by targeting vascular endothelial cells (ECs) [5] and is therefore likely to result in EC activation. In addition to the influence of the drug, ample evidence suggests that inflammatory cytokines are a major promoter of EC activation. In other cancers, the malignant clone induces a cytokine environment that serves as the pivotal background of thromboembolic risk of patients [6]. Similarly, in MM, high levels of inflammatory cytokines have been found, with interleukin-6 (IL-6) and tumor necrosis factor (TNFα) being the most important [7]. We therefore hypothesized that a cytokine environment is also responsible for EC activation, further influencing the thrombogenic potential in MM patients.

Accordingly, we propose that the cytokine environment in MM plus Thal is able to double the risk of VTE in patients with MM. To test

Abbreviations: Thal, Thalidomide; VTE, venous thrombosis; Dex, dexamethasone; vWF, von Willebrand factor; ECs, endothelial cells; IL-6, interleukin-6; TNFα, tumor necrosis factor; HUVECs, human umbilical vein endothelial cells; DMSO, dimethyl sulfoxide; FITC, Fluorescein isothiocyanate; APC, allophycocyanin; PS, phosphatidylserine; TF, tissue factor; PBS, phosphate-buffered saline; EPCR, endothelial protein C receptor; APC, Human activated protein C; TM, thrombomodulin.

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http://dx.doi.org/10.1016/j.thromres.2015.03.019
0049-3848/

this hypothesis, we cultured human umbilical vein endothelial cells (HUVECs) in the presence of MM serum to mimic the cytokine environment of MM and investigate the effect of MM serum and Thal on procoagulant activity, as well as activation of the anticoagulant endothelium protein C. To gain further insight into this mechanism, the roles of cytokine activation were investigated in vitro by adding anti-human TNF and IL-6R monoclonal antibodies to HUVECs incubated in the presence of MM serum.

Subjects, Materials and Methods

Study Subjects

This study was approved by the ethics committee of the Harbin Medical University, and each participant signed an informed consent form. The patients included 20 newly diagnosed or refractory cases of MM who were admitted to the First Affiliated Hospital of Harbin Medical University between July 2011 and September 2013. The exclusion criteria were the following: receiving any drug known to affect hemostasis; presence of inherited thrombophilias and previous history of VTE; and associated disease, including antiphospholipid syndrome, chronic renal disease, heart disease, malignant or systemic disease, diabetes, acute infection, immobilization, surgery, hereditary thrombophilia and hyperviscosity, among other conditions. Ten healthy subjects were included as normal controls. The clinical characteristics of the patients with MM and the controls are shown in Table 1.

Blood samples were collected prior to therapy. Whole blood was collected from different people into a Vacutainer tubes. Serum was prepared by centrifugation at 2000 g for 10 min at 24 °C. Aliquots of 1.0 ml of serum sample were frozen at −80°C and utilized within 1 month.

Materials

Thal (±) and Dex were purchased from ASA (Sigma-Aldrich, Buchs, Switzerland). Thal was dissolved in dimethyl sulfoxide (DMSO) and added to media to reach a final concentration of 0.5 μM. Dex was dissolved in ethanol and added to media to reach a final concentration of 1 mM. The final concentration of DMSO and ethanol was 0.05% in all experiments. Anti-IL-6R and anti-TNFα antibodies were acquired from Abcam (Cambridge, MA, USA). They were first dissolved in DMSO at a 100 μg/ml concentration, then further diluted in culture medium at the time of treatment. The final concentration of anti-IL-6R antibody was 1 ng/ml and that of anti-TNFαantibody was 10 ng/ml. The concentrations of these agents were selected according to previous reports [8–10]. Fluorescein isothiocyanate (FITC)-labeled thrombomodulin (TM), IgG1-FITC, annexin V-FITC, allophycocyanin (APC)-annexin V, IgG-APC and phycoerythrin (PE)-labeled endothelial protein C receptor (EPCR) were obtained from Becton Dickinson (San Jose, CA, USA). Human activated protein C (APC) and protein C (PC) were obtained from Haematologic Technologies, Inc. (Burlington, VT, USA). Antithrombin was obtained from Uscn (Uscn Life Science, Tokyo, Japan). FITC-labeled tissue factor (TF) was obtained from American Diagnostics Inc. The chromogenic substrates S-2238 and S-2366 were obtained from Chromogenix (Italy).

Table 1
Clinical characteristics of patients with MM and healthy controls.

	Patients(MM)	Healthy controls
N	20	10
Age	52 (45-61)	50 (42-60)
Females (%)	35	40
Leukocytes ($\times 10^9$/L)	5.43 (2.65-14.4)	6.0 (4.8-8.6)
Erythrocytes ($\times 10^{12}$/L)*	3.1 (1.39-4.34)	4.4 (3.68-5.13)
Hemoglobin (g/L)*	87.4 (48.7-132.4)	113.4 (106-125)
Platelet ($\times 10^9$/L)	130 (21-258.4)	163.4 (100-209)
PT (s)	11.8 (10.3-13.4)	12.8 (10.8–15.1)
APTT (s)	30.6 (18-55.5)	27.2 (22.1–32.4)
D-D (μg/L)*	39 (0.1-457)	115 (75–199)
IgG (g/L)*	27.08 (2.2-75.5)	9.46 (7.6-12.7)
IgA (g/L)*	11.16 (0.228-42.7)	2.71 (1.2-3.7)
IgM (g/L)*	0.315 (0.16-1.45)	0.852 (0.4-1.2)
β2-MG (mg/L)*	8.44 (1.39-15.49)	2.25 (1.7-3.4)
Bence-Jones proteins k (mg/L)*	64.23 (6.28-81.3)	5.3 (3.5-7.8)
Bence-Jones proteins L (mg/L)*	2033 (3.47-14500)	3.8 (2.9-4.6)
Kappa light chain (g/L)	9.3 (0.79-27.2)	4.4 (3.2-5.7)
lambda light-chain (g/L)	737 (0.64-6390)	3.8 (1.2-5.8)
Albumin (g/L)*	31.3 (19.1-45.9)	38.9 (34.3-43.6)
vWF (%)*	263.1 (171.6-285.4)	93.75 (58-146.2)

Data are presented as the median (range) or number (in percent). *$p < 0.05$ versus controls.

Cell Culture

HUVECs were purchased from Clonetics Corporation (San Diego, CA, USA). The cells were grown in ECM medium and maintained at 37°C in a humidified incubator in an atmosphere of 5% CO2/95% air. Cells were seeded in six-well plates at an initial density of 1×10^4 cells/cm2, cultured to sub-confluent conditions and experiments were typically performed on day three. The HUVECs were divided into five experimental groups: untreated, serum alone-treated, drug alone-treated, serum and drug treated and inhibitor-treated groups. In the serum alone-treated group, HUVECs were treated with 15% individual normal serum (serum from healthy subjects) or MM serum for 12 h; In the drug alone-treated group, HUVECs were treated with Thal and Dex alone or in combination for 12 h. In the serum and drug treated group, HUVECs were pretreated with 15% individual normal serum or MM serum for 12 h, and then treated with Thal and Dex alone or in combination for 12 h. In the inhibitor-treated group, HUVECs were pretreated with anti-IL-6R or anti-TNFα antibodies for 6 h and 15% individual MM serum for 6 h, and then treated with Thal and Dex alone or in combination for 12 h. For all experiments, HUVECs were seeded in 6-well culture plates. All experiments were performed on HUVECs after 4-8 passages. All cell culture results shown are based on three individual experiments at least.

Flow Cytometric Analysis of Cell Surface Phosphatidylserine (PS) and TF on HUVECs

HUVECs were detached with trypsin-EDTA and enumerated using flow cytometry as described previously. HUVECs (2×10^6) were incubated with 100 μl of calcium-containing binding buffer for 10 min at room temperature, and 5 μl of annexin V-APC/FITC, TF-FITC, or isotype-matched control antibodies were then added. The samples were incubated for 20 min at room temperature in the dark. After incubation, 400 μl of calcium-containing binding buffer was added to the samples. The samples were analyzed using a FACSCalibur flow cytometer (Becton Dickinson, San Jose, CA, USA) that measured 10000 events per sample in duplicate. Data acquisition was performed using CELLQUEST PRO software (San Jose, CA, USA).

Thrombin Generation Assays

Thrombin generation assays were performed as previously described [11]. Briefly, detached HUVECs (2×10^6) were washed twice with 1 ml of PBS. The ECs were resuspended in defibrinated plasma previously treated with batroxobin. The mixture was then prewarmed (37 °C) for 10 min. At $t = 0$, 100 μl of 0.05 mmol/l $CaCl_2$ in PBS was added to initiate thrombin generation. Every 2 min, a 25 μl aliquot was removed from the reaction mixture and added to 125 μl of prewarmed (37 °C) PBS containing the chromogenic substrate. After 3 min, 100 μl of 1 mM citric acid was added to stop the conversion of S2238, and the absorbance was measured at 405 nm using a spectrophotometer (Molecular Devices, Union City, CA, USA). To convert the observed optical density (OD) into the thrombin concentration,