The Comparison of Maximal Platelet Aggregation in the Presence of Disperse Primary and Monolayer Secondary HUVEC Exposed to Testosterone in High Glucose Medium

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Abstract

To explore in vitro models in examining the influence of testosterone (T) to platelet activation through endothelial cells in hyperglycemia condition. Disperse primary and monolayer secondary human umbilical vein endothelial cells (HUVEC) in high glucose (HG) medium were exposed to T. Maximal platelet aggregation in the presence of HUVEC was measured using turbidimetric method. Maximal platelet aggregation in the presence of disperse primary HUVEC exposed to 0, 1, 10, and 100 nM of T were 69.4%, 65.4%, 67.6%, and 62.05%, whereas of

monolayer secondary HUVEC were 46.5%, 43.9%, 48.1%, and 37.45%, respectively. Maximal platelet aggregation was significantly lower ($p = 0.0001$) in disperse primary HUVEC than in monolayer secondary HUVEC. The two methods were moderately correlated ($r = 0.06$; $p = 0.079$) in terms of endothelial activation.Maximal platelet agregation in the presence of disperse primary HUVEC is higher than, but correlated to, of monolayer secondary HUVEC, which is exposed to T in HG medium.

Key words: platelet aggregation, endothelial cells, testosterone, high glucose, in vitro.

Introduction

Cardiovascular (CV) disease has high morbidity and high mortality. Male sex has been considered as one of traditional risk factors for CV disease. Recent literature shows that androgen hormones, chiefly testosterone (T), play roles in CV disease. The detection of androgen receptors in the CV system indicates that the heart and vessels are organ targets for T [1,2].

To investigate the influence of T to the CV disease, in vitro experimental studies are needed. As endothelial dysfunction contributes to the development CV disease [3], many studies have been done in endothelial cell culture to get insight into the mechanism of CV disease. Among models for endothelial cell culture, human umbilical vein endothelial cells (HUVEC) has been developed and used extensively [4].

One of the functions of endothelial cells is to maintain blood flow by inhibiting thrombosis. Platelets adhesion and aggregation has been considered as the early events in thrombus development [5]. Several papers have reported that endothelial cells culture could inhibit maximal platelet aggregation through thromboregulators synthesis and release, such as nitric oxide or endothelium derived relaxing factor and prostacyclin [6-8].

Hyperglycemia is hallmark of Diabetes Mellitus (DM). Moreover, it induces endothelial dysfunction and contributes to the development of CV disease [9]. Low T level (hypogonadism) was frequently found among type 2 DM patients [10].

Materials and Methods

All experiments were carried out with ethical permission for the study was obtained by Medical and Health Research Ethics Committee (MHREC), Faculty of Medicine Gadjah Mada University, Yogyakarta, Indonesia, with reference № KE/FK/477/EC.

Materials

Medium 199 powder (Gibco), DMEM high glucose (Gibco), Penicillin Streptomycin (Gibco), Fungizone (Gibco), Fetal Bovine Serum (Caisson), Trypsin 0.25%-EDTA (Gibco), Testosterone (T) powder (Nacalai), D-Glucosa powder (LPPT UGM), Dulbecco's Phosphate Buffer Saline (Gibco).

Isolation of endothelial cells

Source of endothelial cells was umbilical vein of the umbilical cord from delivery mothers in midwifery clinics in Yogyakarta. After the umbilical cord was cut from the placenta at length about 20 cm, it was collected in the phosphate buffer saline (PBS), which was supllemented with Penicillin 100 IU/mL and Streptomycin 100 µg/mL. When the cord could not be processed directly in the culture laboratory after delivery, it was stored at 4^0C.

Endothelial cells were isolated using enzymatic method based on a previous work done by Tanaka [11]. Under a biosafety cabinet level 2, the umbilical cord was removed from the cord buffer. Using povidone iodine 10%, the outside of the cord was cleaned with sterile kassa. As the lumen of umbilical vein and arteries were identified, a cannula was inserted into the umbilical vein and tightened with a hemostatic clamp. To remove blood clots inside the vein, ringer lactate solution was injected to the lumen using spuit injection 10 cc for several times. After the vein was free from serum, warm trypsin 0.25%-EDTA solution was injected and another edge of the cord was also clamped with a hemostatic clamp. After the vein was distended, the injection of the enzyme was stopped and the cord was incubated in warm PBS Pen Strep 1% solution for two minutes. Then the clamp was loosened and the effluent was collected into a sterile conical tube

with 1 mL FBS inside for antitrypsin. To ensure that all the disaggregated endothelial cells was collected, the vein was washed with PBS Pen Strep 1% solution and save together with the enzymatic effluent in the same conical tube. The effluent was centrifuged at 2000 rpm for ten minutes. Then the supernatant was discharged and the pellet was resuspended with 4 cc growth medium, i.e. Medium 199 or DMEM high glucose, which supplemented with Penicillin 100 IU/mL-Streptomycin 100 µg/mL, Fungizone 0.5% and FBS 10%. DMEM high glucose was used for dispersed primary HUVEC, whereas M199 added with D-glucose was used for monolayer secondary HUVEC. The cell suspension was plated at a tissue culture dish (TCD) coated with gelatin 2%. The dish was then incubated at 37^0 C and 5% CO_2 for 24 hours. In the following day, the old medium was removed and the dish was carefully washed with PBS Pen Strep 1% to rid of erythrocytes. The dish was then provided with fresh growth medium. Using an inverted microscope, the endothelial cells attached to the surface of the dish in the form of colonies. They were seen as elongated polygonal cells, with one nucleus and distinct cell membrane. The dish was incubated at 37^0 C and 5% CO_2 and the medium was changed every two days. At the 7th-9th day, the endothelial cells reached 80% confluences.

Disperse primary HUVEC

The subconfluent primary HUVEC was harvested using trypsin 0.25%-EDTA. Briefly, the TCD containing primary HUVEC was brought from the incubator and observed under inverted microscope. As the monolayer HUVEC reached 80% confluencies, the TCD was put on the BSC level 2 and the old medium was discarded. The monolayer HUVEC was washed with warm PBS solution twice to remove traces of serum. Then covered the monolayer with 450 µL of trypsin 0.25%-EDTA and incubated at 37^0 C for 3 minutes. The cells was observed under inverted microscope to see whether they have dettached from the surface. Gentle tap at the side would help cells dettachment. DMEM high glucose with FBS 10% as antitrypsin was added to the TCD. The cells suspension was pipetted and collected into a 15 mL conical tube. The cells suspension was centrifuged at 2000 rpm for 5 minutes. The supernatant was discarded and the cells pellet was resuspended with 1 mL of complete medium. The endothelial cells were counted

and then divided into four microtubes at density 6 x 10^3 cells/tube in DMEM high glucose. Themicrotubes were added with T as the following doses: 0 nM, 1 nM, 10 nM, and 100 nM, consecutively, and incubated at 37^0 C for 30 minutes. After incubation, the microtubes were centrifuged and the supernatant was discarded until it remains at about 100 µL.

Secondary monolayer HUVEC

As the primary HUVEC reached 80% confluences, the monolayer endothelial cells was trypsinized with trypsin 0.25%-EDTA. Following cell counts, the endothelial cells were plated in gelatin-coated 24-well dishes at density 3 x 10^4 cells/mL At 60-70% confluences, the subculture endothelial cells were treated with M199 high glucose with T at doses 0 nM, 1 nM, 10 nM, and 100 nM for 24 hours. The following day, the treatment medium was discarded and the monolayer was trypsinized. The endothelial cell suspension was centrifuged and as supernatant was discarded, the cell pellet was resuspended in serum free medium.

Platelet preparation and aggregation

Platelet rich plasma (PRP) was prepared from whole blood of healthy male subjects. The blood was removed from vena cubiti with vacutainers containing 3.1% sodium citrate in ratio 9:1. The blood was centrifuged at 900 rpm for 15 minutes. The supernatant, i.e. PRP, was collected. The remaining blood was centrifuged at 3000 rpm for 15 minutes and the supernatatnt, as platelet poor plasma (PPP), was collected also.

Maximal platelet aggregation was measured using an aggregometer machine from Helena Lab, with adenosine diphosphate (ADP) as platelet agonist. To obtain ADP at dose 5 µM, the ADP stock at dose 20 µM was diluted with normal saline at ratio 1:3. Then, 400 µL of PRP was incubated with 50 µL of endothelial cell suspension in cuvettes at room temperature for 3 minutes. The cuvette was subsequently put in the machine, stirred and added with 50 µL of ADP 5 µM. Maximal platelet aggregation was measured for 10 minutes based on light transmission. A batch of maximal platelet aggregation measurement consisted of 4 cuvettes. A cuvette containing

PRP was used as a standard (100% transmittance), and an empty cuvette was used as blank (0% transmittance).

Data analysis

To examine whether maximal platelet aggregation in the presence of disperse primary HUVEC and of monolayer secondary HUVEC treated with T in high glucose (HG) medium was correlated, data was analyzed using Pearson correlation. Maximal platelet agregation in the presence of disperse primary HUVEC was compared to of monolayer secondary HUVEC by independent t-test. *p* value < 0.05 was considered significant.

Results and Discussion

Maximal platelet aggregation in the presence of disperse primary HUVEC and of monolayer secondary HUVEC treated with T in HG medium (Table 1) was moderateley correlated ($r = 0.06$, *p* value $= 0.079$). However, using independent t-test, maximal platelet aggregation in the presence of disperse primary HUVEC was significantly higher than of monolayer secondary HUVEC treated with T in HG medium ($66.11 \pm 4.26\%$ *vs*. $43.4 \pm 6.27\%$, *p* $= 0.0001$).

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This study shows that disperse primary and monolayer secondary endothelial cells treated with T in HG medium exert influence to maximal platelet aggregation in parallel fashion. The aim of this study is to explore whether T treatment in HG medium to disperse primary HUVEC

and monolayer secondary HUVEC exerted similar influence to maximal platelet aggregation.

Table 1. Comparison of maximal platelet aggregation in the presence of disperse primary and monolayer secondary endothelial cells treated with testosterone in high glucose medium

Group	Maximal platelet aggregation (%) in the presence of			
	Disperse primary HUVEC		Monolayer secondary HUVEC	
	Mean	St.dev.	Mean	St.dev.
T0	69.4	1.56	46.5	10.7
T1	65.4	3.39	43.9	1.41
T10	67.6	3.68	48.1	-
T100	62.1	6.29	37.5	2.33

Note: T0 = testosterone 0 nM, T1 = testosterone 1 nM, T10 = testosterone 10 nM, T100 = testosterone 100 nM. St.dev. = standard deviation.

Several studies had investigated the influences of T to endothelial cells in some aspects, such as apoptosis, expression of cell adhesion molecules, tissue plasminogen activator, and tissue factor. The incubation time varies ranging from 2 hours to 48 hours [12-18]. As reviewed by Liu et al. [1], the influence of T, as well as other steroid hormones, to cell target is mediated by androgen receptor (genomic) or another pathway that is not associated with androgen receptor (non-genomic). One of the differences between the two mechanisms is time duration. Genomic mechanism is mediated through transcription and translation of protein related with androgen related elements located within the DNA of target nuclear genes, whereas non-genomic mechanism is mediated by induction of second messenger signal transduction cascades, such as protein kinase A, protein kinase C, and calcium. Therefore, genomic effect of T is responsible for long term response, whereas non-genomic is for short term response. Thus, time incubation of T exposure to endothelial cells determines whether T plays role through genomic or non-genomic mechanism. In this study, T exposure time to disperse primary endothelial cells is much

shorter than to monolayer secondary endothelial cells (30 minutes *vs*. 24 hours, respectively). Therefore, the pattern of maximal platelet aggregation in the presence of post-treated endothelial cells culture in this study reflects both non-genomic (in disperse primary HUVEC) and genomic effect (in monolayer secondary HUVEC). Indeed, the passage of unconjugated T into cell culures (hamster fibroblast and rat hepatoma tetraploid cells) occurs by a simple diffusion through the lipid bilayer of the plasma membrane, with permeability coefficient 0.66×10^{-4} cm sec^{-1}, partition coefficient 3.9 to 61, and diffusion coefficient $1.1. \times 10^{-12}$ to 1.7×10^{-11} $cm^2 sec^{-1}$. Therefore, $t_{1/2}$ of equilibration of testosterone in those cells are fast, i.e. 20 to 25 seconds [19]. To our knowledge, this study is the first that reports the effect of non-genomic effect of T by exposing it to disperse primary culture of endothelial cells since previous studies explored non-genomic effect of T by exposing it to monolayer endothelial cells [12, 15, 17].

This study also shows that the magnitude of maximal platelet aggregation in the presence of monolayer secondary endothelial cells treated with T in HG medium is significantly lower than of disperse primary endothelial cells. The difference in magnitude of platelet aggregation may relate to the amount of endothelial cells between disperse primary and monolayer secondary HUVEC used in this experiment (6×10^3 cells/group *vs*. 3×10^4 cells/group, respectively). However, apart from this disparity, the lower magnitude of platelet aggregation in the monolayer secondary endothelial cells as compared to the disperse primary endothelial cells may reflect two things. First, genomic action of T to endothelial cells in inhibiting platelet aggregation is greater than non-genomic effect of T. Second, as monolayer secondary HUVEC is more homogenous than disperse primary HUVEC, the capacity of endothelial cells to counter platelete aggregation is greater in monolayer secondary HUVEC than in disperse primary HUVEC.

Conclusion

Exposing testosterone in high glucose medium to disperse primary and monolayer secondary endothelial cells exert similar influence to platelet aggregation. Both can be used as models to examine the role of testosterone in platelet aggregation through endothelial cells.

Duration of exposure determines whether non-genomic and/or genomic action of testosterone will play role.

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Disclosure

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References

[1] Liu P, Death AK and Handelsman DJ. (2003) Androgens and Cardiovascular Disease. Endocr Rev. 24(3), 313-340.

[2] Wu FCW and von Eckardstein A. (2003) Androgens and coronary artery disease. Endocr Rev. 24, 183-217.

[3] Cines DB, Pollak ES, Buck CA, Loscalzo J, Zimmerman GA, McEver RP et al. (1998) Endothelial Cells in Physiology and in the Pathophysiology of Vascular Disorders. Blood. 91, 3527-3561.

[4] Nachman RL and Jaffe EA. (2004) Endothelial cell culture: beginnings of modern vascular biology. J. Clin. Invest. 114, 1037–1040.

[5] Galley HF and Webster NR. (2004) Physiology of the endothelium. Br J of Anaesthesia. 93 (1), 105-113.

[6] Lagarde M, Felisi O, Boukhchache, D, Sicard, B and Dechavanne M. (1984) Relationship between cyclic AMP and thromboxane formation in platelet-endothelial cell interactions. Thromb Res. 25, 141-147.

[7] Nordoy A, Svensson B and Hoa JC. (1978) The inhibitory effect of human endothelial cell monolayers on platelet reactions and its inhibition by aspirin. Thromb Res. 12, 597-608.

[8] Altorjay I, Kirchmaier CM and Breddin HK. (1999) Changes of spontaneous and induced platelet aggregation in the presence of a human endothelial cell monolayer. Thromb Res. 55, 69-77.

[9] Popov D. (2010) Endothelial cell dysfunction in hyperglycemia: Phenotypic change, intracellular signaling modification, ultrastructural alteration, and potential clinical outcomes. Int J Diabetes Mellitus. 2, 189–195.

[10] Mijares AH, Malpartida KH, Izquierdo ES, Bañuls C, Rocha M, Martínez MJG. et al. (2010) Testosterone levels in males with type 2 diabetes and their relationship with cardiovascular risk factors and cardiovascular disease. J Sex Med 7, 1954–1964.

[11] Tanaka A. (1994) Primary Culture of HUVEC. Department of Obstetric and Gynecology, Kobe University School of Medicine.

[12] Hatakeyama H, Nishizawa M, Nakagawa A, Nakano S, Kigoshi T and Uchida K. (2002) Testosterone inhibits tumor necrosis factor-α-induced vascular cell adhesion molecule-1 expression in human aortic endothelial cells. FEBS Lett. 530, 129-132.

[13] Ling S, Dai A, Williams MRI, Myles K, Dilley RJ, Komesaroff PA et al. (2002) Testosterone (T) enhances apoptosis-related damage in human vascular endothelial cells. Endocrinology. 143(3), 1119-1125.

[14] Mukherjee TK, Dinh H, Chauduri G and Nathan L. (2002) Testosterone attenuates expression of vascular cell adhesion molecule-1 by conversion to estradiol by aromatase in endothelial cells: Implications in atherosclerosis. Proc Natl Acad Sci USA, 99(6), 4055-4060.

[15] Zhang X, Wang LY, Jiang TY, Zhang HP, Dou Y, Zhao JH. et al. (2002) Effects of testosterone and 17-β-estradiol on TNF-α-induced E-selectin and VCAM-1 expression in

endothelial cells: analysis of the underlying receptor pathways. Life Sciences. 17, 15-29.

[16] Jin H, Lin J, Mei YF, Peng G, Tan X, Wang DM. et al. (2007) Physiological testosterone stimulates tissue plasminogen and activator and tissue factor pathway inhibitor and inhibits plasminogen activator inhibitor type 1 release in endothelial cells. Biochem Cell Biol. 85, 246-251.

[17] Goglia L, Tosi V, Sanchez AM, Flamini MI, Fu XD, Zullino S. et al. (2010) Endothelial regulation of eNOS, PAI-I, and t-PA by testosterone and dihydrotestosterone in vitro and in vivo. Mol Hum Reprod. 16(10), 761-769.

[18] Campelo AE, Cutini PH and Massheimer VL. (2012) Testosterone modulates platelet aggregation and endothelial cell growth through nitric oxide pathway. J Endocrinol. 213, 77-87.

[19] Giorgi EP and Stein WD. (1981) The transport of steroids into animal cells in culture. Endocrinology. 108(2), 688-697.

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