



UNIVERSITI PUTRA MALAYSIA

***MECHANISMS OF INTERFERON-GAMMA (IFN- γ)-INDUCED
HYPERPERMEABILITY CHANGES IN HUMAN UMBILICAL VEIN
ENDOTHELIAL CELLS***

NG CHIN THENG

FPSK(P) 2017 9



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By

NG CHIN THENG

Thesis Submitted to the School of Graduate Studies,
Universiti Putra Malaysia, in Fulfilment of the Requirements for the Degree of
Doctor of Philosophy

January 2017

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Abstract of thesis presented to the Senate of Universiti Putra Malaysia in fulfilment of the requirement for the degree of Doctor of Philosophy

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January 2017

Chair : Zuraini Ahmad, PhD

Faculty : Medicine and Health Sciences

Endothelial dysfunction, characterized by increased endothelial permeability, is the initiating step in the pathogenesis of vascular diseases such as atherosclerosis. Interferon-gamma (IFN- γ), a pro-inflammatory cytokine, has been reported to impair the endothelial barrier and thus, increases vascular permeability. However, the mechanism by which IFN- γ disrupts the endothelial barrier has never been clarified. Therefore, this study aimed to evaluate the underlying mechanisms of IFN- γ -induced hyperpermeability changes using human umbilical vein endothelial cells (HUVECs). HUVECs were used as a model system to study permeability changes because inflammatory events are commonly occurs in postcapillary venules *in vivo*. As a preliminary step, the HUVECs viability was determined using MTT and ATP assays. Permeability changes were assessed using *in vitro* permeability assay kits. Localization of F-actin, caldesmon, β -catenin and vascular endothelial cadherin (VE-cadherin) was studied using confocal microscope. Total protein expressions of β -catenin, VE-cadherin, F/G-actin, p38 MAP kinase, phosphorylated-p38 MAP kinase (p-p38 MAP kinase), caldesmon and phosphorylated-caldesmon (p-caldesmon) were performed using Western blot analysis. Protein expressions of β -catenin and VE-cadherin in different cell compartments were studied using subcellular protein fractionation kit. The interactions of caldesmon to actin and myosin were studied using a co-immunoprecipitation assay. The levels of nitric oxide (NO) and cyclic guanosine monophosphate (cGMP) were detected using Griess assay and enzyme-linked immunosorbent assay (ELISA), respectively. The present study showed that IFN- γ increased HUVECs permeability in a biphasic manner. The hyperpermeability changes may be associated with actin remodeling and alteration of adherens junctions (AJs). In the first phase, IFN- γ caused cell rounding and peripheral actin bands, which may be regulated by caldesmon phosphorylation and dissociation of actin with caldesmon. Besides, IFN- γ induced discontinuous AJs formation without altering the AJs expression level. On the other hand, the second phase of increased permeability involves cell elongation and stress fiber formation, which may be regulated by F-actin hyperpolymerization. Besides, IFN- γ induced linearized AJs, and downregulated the AJs expression level in membrane and cytoskeleton fractions. The results showed that

IFN- γ activated p38 MAP kinase in the signaling pathway. However, p38 MAP kinase only regulated the first phase of IFN- γ -mediated increased permeability, and F-actin remodeling. Besides, NO partially regulated the IFN- γ -induced HUVECs hyperpermeability and this was independently of cGMP. In summary, the study enhances the current knowledge on the mechanism of IFN- γ in inducing endothelial dysfunction. The mechanisms underlie IFN- γ -mediated HUVECs hyperpermeability may involve F-actin remodeling and alteration of AJs structure and expression, suggesting that actin cytoskeleton and AJs may serve as the potential therapeutic targets for prevention of the endothelial dysfunction mediated by IFN- γ . The p38 MAP kinase and NO are not the primary regulator for the regulation of IFN- γ -induced endothelial barrier impairment.

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia sebagai memenuhi keperluan untuk ijazah Doktor Falsafah

**MEKANISME PERUBAHAN HIPERKEBOLEHTELAPAN ARUHAN
INTERFERON-GAMMA (IFN- γ) PADA SEL ENDOTELIAL VENA
UMBILIKAL MANUSIA**

Oleh

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Disfungsi endotelial yang dicirikan sebagai peningkatan ketelapan endotelial merupakan langkah aksi pembuka dalam patogenesis bagi penyakit vaskular seperti aterosklerosis. Interferon-gamma (IFN- γ) ialah salah satu sitokin pro-keradangan yang telah dilaporkan boleh mengganggu integriti sekatan sel endotelial dan meningkatkan ketelapan vaskular. Numun demikian, mekanisme mengenai IFN- γ bagi gangguan sekatan sel endotelial tak pernah dijelaskan. Oleh itu, kajian ini bertujuan untuk menyiasat mekanisme perubahan hiperkebolehtelapan aruhan IFN- γ dengan menggunakan sel endotelial vena umbilikal manusia (HUVECs). HUVECs telah digunakan sebagai model bagi menilai perubahan pada ketelapan kerana keradangan biasanya berlaku pada venul pascakapilari. Kebolehhidupan sel HUVEC telah ditentukan dengan menggunakan assai MTT dan ATP. Kebolehtelapan endotelia telah dinilai dengan menggunakan kit assai kebolehtelapan secara *in vitro*. Penyetempatan F-aktin, caldesmon, β -catenin dan VE-cadherin telah dikaji dengan menggunakan mikroskop mengimbas sefokus. Jumlah ekspresi protein β -catenin, VE-cadherin, F-/G-aktin, p38 MAP kinase, p38 MAP kinase terfosforil (p-p38), caldesmon dan caldesmon terfosforil (p-caldesmon) telah dikaji dengan menggunakan kaedah pemendapan Western. Ekspresi protein β -catenin dan VE-cadherin pada pangsa sel yang berbeza telah dikaji dengan menggunakan kit fraksinasi subsel. Interaksi antara caldesmon-aktin dan caldesmon-miosin telah dikaji dengan menggunakan assai kopengimunomendakan. Tahap nitrik oksida (NO) dan guanine monofosfat siklik (cGMP) diukur dengan menggunakan assai Griess dan ELISA. Kajian ini menunjukkan bahawa IFN- γ meningkatkan kebolehtelapan endotelial secara dwifasa. Hiperkebolehtelapan yang dicerap berkait rapat dengan pembetukan semula aktin dan perubahan pada simpangan adherens (AJs). Dalam fasa pertama, IFN- γ menyebabkan pembulatan sel dan pembentukan jalur aktin di sekeliling sel, dan perubahan ini mungkin dikawalkan oleh pemfosforilan caldesmon dan pemisahan aktin dengan caldesmon. Selain itu, IFN- γ mencetuskan pembentukan AJs tidak berterusan tanpa mengubah tahap ekspresi AJs. Sebaliknya, peningkatan ketelapan pada fasa kedua melibatkan pemanjangan sel dan pembentukan fiber tekanan, dan semua perubahan ini mungkin dikawal oleh hiperpolimerisasi aktin. Selain itu, IFN- γ juga mendorong pembentukan AJs terlinear

dan mengurangkan ekspresi AJs pada pecahan-pecahan membran dan sitoskeleton. Keputusan menunjukkan IFN- γ mengaktifkan p38 MAP kinase dalam lata isyarat molekul. Tetapi, p38 MAP kinase hanya mengawal pembentukan semula aktin dan peningkatan ketelapan yang dicetuskan oleh IFN- γ dalam fasa pertama. Selain daripada itu, NO juga terlibat dalam mengawal peningkatan ketelapan yang dicetuskan oleh IFN- γ , dan ini tidak bersandar pada cGMP. Kesimpulannya, kajian ini meningkatkan pemahaman semasa mengenai mekanisme pencetusan dysfungsi endotelial oleh IFN- γ . Mekanisme IFN- γ yang mencetuskan peningkatan ketelapan mungkin terlibat pembentukan semula aktin dan perubahan pada struktur dan expresi AJs. Justeru itu, sitoskeleton aktin dan AJs mungkin merupakan sasaran terapeutik baharu bagi mencegah dysfungsi endotelial yang dicetuskan oleh IFN- γ . Keputusan juga menunjukkan p38 MAP kinase dan NO bukan faktor utama yang menjelaskan fungsi pengadang sel endotelial yang diaruhkan oleh IFN- γ .

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I certify that a Thesis Examination Committee has met on 26 January 2017 to conduct the final examination of Ng Chin Theng on her thesis entitled “Mechanisms of Interferon-Gamma (IFN- γ)-Induced Hyperpermeability Changes in Human Umbilical Vein Endothelial Cells” in accordance with the Universities and University Colleges Act 1971 and the Constitution of the Universiti Putra Malaysia [P.U.(A) 106] 15 March 1998. The Committee recommends that the student be awarded the Doctor of Philosophy.

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LIST OF ABBREVIATIONS

AJ	Adherens junction
ERK	Extracellular signal-regulated kinase
FITC	Fluorescein isothiocyanate
ICAM	Intercellular adhesion molecule
IFN	Interferon
IL	Interleukin
JAK	Janus kinase
JAM	Junction adhesion molecule
JAM	Junctional adhesion molecule
MAPK	Mitogen-activated protein kinase
MLC	Myosin regulatory light chain
NF-κB	Nuclear factor-kappa B
NO	Nitric oxide
NOS	Nitric oxide synthase
p38MAPK	p38 mitogen-activated protein kinase
PAF	Platelet-activating factor
PECAM	Platelet endothelial cell adhesion molecule
PI3K	Phosphoinositol-3'-OH kinase
PKC	Protein kinase C
PMA	Phorbol-12-myristate-13-acetate
RFU	Relative fluorescence unit
ROCK	Rho-associated protein kinase
SOCS	Suppressor of cytokine signaling
STAT	Signal transducer and activator of transcription
TJ	Tight junction
TNF	Tumour necrosis factor
VCAM	Vascular cell adhesion molecule
VVO	Vesiculo-vacuolar organelle

CHAPTER 1

INTRODUCTION

1.1 Justification of the Study

Endothelial dysfunction, characterized by an increase in endothelial permeability, is an early step in the pathogenesis of vascular diseases. Endothelial dysfunction is mainly resulted from disruption of the endothelial barrier function. IFN- γ , a pro-inflammatory cytokine, has been reported to impair the endothelial barrier and causes increased endothelial permeability (Stolpen *et al.*, 1986). Accumulating evidence over the past decade has highlighted the roles of IFN- γ in mediating various types of vascular diseases such as atherosclerosis (Voloshyna *et al.*, 2014). However, the underlying mechanisms by which IFN- γ causes increased endothelial permeability, particularly the roles of cytoskeleton and interendothelial junctions, have never been clarified. In addition, the signaling pathways leading to IFN- γ -induced endothelial hyperpermeability remain unknown. Therefore, understanding of the mechanisms underlie IFN- γ -induced endothelial barrier disruption is important and it could provide novel therapeutic targets that can be further developed to treat IFN- γ -associated vascular diseases.

1.2 Background of the study

Vascular endothelial cells form a thin monolayer called endothelium which encloses the inner surface of the blood vessels, forming a natural protective barrier that separates the bloodstream from underlying tissue. This protective barrier allows the small molecules to pass through the endothelium while restricts the passage of large molecules; this is known as semi-permeable barrier (Vandenbroucke *et al.*, 2008). Under inflammatory condition, the endothelial cells become activated that in turn leads to impairment of the barrier function; this is known as endothelial dysfunction. As a consequence, large amount of solutes and cells escape from bloodstream and enter into underlying tissue; these result in oedema formation, a hallmark of acute inflammation (Murakami and Hirano, 2012).

IFN- γ is a proinflammatory cytokine well known to interfere with viral replication and defense against microbial infection. Due to this reason, the immunoregulatory activity of IFN- γ has been well studied and characterized (Akdis *et al.*, 2011). However, accumulating evidence has shown that IFN- γ is a major cytokine that participates actively in the development of vascular diseases such as atherosclerosis (Voloshyna *et al.*, 2014). Indeed, elevated levels of IFN- γ have been detected in atherosclerotic lesions of atherosclerosis patients and animal models (Voloshyna *et al.*, 2014). However, the impact of IFN- γ in regards to inflammation, particularly endothelial dysfunction, largely remains unknown.

In physiological conditions, the endothelial cells are closely bound to each other through interendothelial junctions such as adherens junctions (AJs) (Gavard, 2009;

Vandenbroucke *et al.*, 2008). The AJs are connected to intracellular actin cytoskeleton via β -catenin linker protein (Yuan and Rigor, 2010b). Besides, the actin stability is regulated by an actin-binding protein known as caldesmon (Mayanagi and Sobue, 2011). The AJs, actin and caldesmon have been recognized as key regulators for the maintenance of barrier function, and therefore disturbances of these molecules will promote the leakage of molecules across the vascular endothelium. Apart from the aforementioned regulators, signaling molecules such as p38 mitogen-activated protein (p38 MAP) kinase (Adderley *et al.*, 2015), nitric oxide (NO) and cyclic guanosine monophosphate (cGMP) (Atochin and Huang, 2010) are also well known to regulate the endothelial barrier function. Given the significance roles of actin, caldesmon, AJs, p38 MAP kinase and NO-cGMP in the regulation of endothelial barrier, a better understanding of the roles of these regulators in regulating the IFN- γ -induced hyperpermeability changes might lead to the development of new pharmacotherapies to mitigate vascular disease progression initiated by IFN- γ .

In this study, HUVECs were selected due to the critical importance of vascular endothelial cells in the development of vascular disease. Importantly, inflammatory events, such as increased permeability, occur predominantly in postcapillary venules *in vivo* (Aird, 2007). Therefore, endothelial cells derived from human vein are more responsive to inflammatory stimuli such as proinflammatory cytokine. Under appropriate culture conditions, HUVECs form a continuous endothelium, characterized by continuous endothelial cell layer and well-formed basement membrane, (Hamilton *et al.*, 2007), yet they can undergo dynamic cellular changes towards agonist (Aird, 2012). Due to these unique features of HUVECs, HUVECs are a suitable model system for the assessment of permeability changes *in vitro*, and the application of IFN- γ was used to mimic the inflammatory conditions in human body.

1.3 Objective

1.3.1 General Objective

The study was to determine the underlying mechanisms of IFN- γ in increasing permeability of human umbilical vein endothelial cells.

1.3.2 Specific Objectives

The specific objectives of this study were:

1. To investigate the effect of IFN- γ in HUVECs permeability.
2. To elucidate the role of cytoskeleton in the regulation of HUVECs permeability following IFN- γ stimulation.
3. To elucidate the regulatory role of cytoskeleton-associated protein on IFN- γ -induced cytoskeletal remodeling.
4. To elucidate the organization of interendothelial junction upon IFN- γ stimulation.

5. To examine the signaling molecules that regulates HUVECs permeability changes, cytoskeleton remodeling and alteration of interendothelial junction upon IFN- γ stimulation.

1.4 Hypotheses

IFN- γ will impair the HUVECs barrier function by inducing endothelial permeability changes, which will involve cytoskeleton, cytoskeleton-associated protein and interendothelial junction. The molecular mechanisms activated by IFN- γ will involve p38 MAP kinase and NO-cGMP pathways.

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