International Immunopharmacology 10 (2010) 1552–1559

Contents lists available at ScienceDirect

International Immunopharmacology

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

Blockade of PKC-beta protects HUVEC from advanced glycation end products induced inflammation

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ARTICLE INFO

Article history:
Received 7 July 2010
Received in revised form 15 August 2010
Accepted 10 September 2010

Keywords:
Endothelial cell
ICAM-1
Macrophage migration
Oxidative stress
TGF-beta1
PKC-beta

ABSTRACT

Advanced glycation end products (AGEs) have been recognized as a pivotal inducer in diabetes and kinds of aging-related vasculopathy. Endothelial dysfunction and inflammatory cells adhesion to endothelium have been regarded as important and early factors in the pathogenesis of vascular complications in diabetic patients. Owing to the key role of PKC-beta in AGEs-induced vascular dysfunction, we investigated effects of blocking PKC-beta by LY333531 on macrophage adhesion to HUVEC and the related mechanism. Transwell HUVEC-macrophage co-culture system was established to evaluate macrophage migration and adhesion ability. Immunocytochemistry was applied to examine TGF-beta1, ICAM-1 and RAGE protein expressions by SABC or SABC-AP method; mRNA expression of TGF-beta1, ICAM-1 and RAGE was determined by real-time RT-PCR. SOD and MDA levels in culture supernatant were detected. We found that LY333531 significantly reduced AGEs-induced macrophage adhesion to HUVEC. Blockade of PKC-beta strikingly decreased HUVEC TGF-beta1 and ICAM-1 expression in both protein and mRNA levels, RAGE protein level was also down-regulated. Furthermore, the anti-oxidative stress index, SOD/MDA was dramatically elevated on LY333531 application. Therefore we conclude that LY333531 can reduce AGEs-induced macrophage adhesion to endothelial cells and relieve the local inflammation, this was realized by its effect on decreasing inflammatory cytokines' expression and increasing cell anti-oxidative ability.

1. Introduction

Endothelium is the major vector in angiogenesis [1], and endothelial dysfunction is regarded as an important and early factor in the pathogenesis of atherothrombosis [2] and vascular complications [3] in diabetic patients. The complications associated with diabetic vasculopathy are commonly grouped into two categories: micro- and macro-vascular complications. In diabetes, macro-vascular disease is the commonest cause of mortality and morbidity and is responsible for high incidence of vascular diseases such as stroke, myocardial infarction and peripheral vascular diseases [4]. However, the morbidity associated with diabetic micro-vascular disease, including retinopathy, neuropathy, nephropathy, and limb ischemia, is staggering [5]. Infiltration of macrophages in the glomeruli and interstitium is one of the characteristic features of diabetic nephropathy, in addition to ECM expansion and interstitial fibrosis [6]. Recruitment of monocyte/macrophage and lymphocytes from the peripheral blood to the endothelial cells (ECs) is an early and central event in vascular dysfunction development [5,7] and experimental diabetes [22], thus inflammation plays a pivotal role in the initiation of this process.

Advanced glycation end products (AGEs) have been widely studied in its key role in promoting vascular dysfunction and diabetes development [5]. AGEs impair vessel functions via the receptor for advanced glycation end products (RAGE) [8]. The activation of RAGE may induce a series of inflammatory process, such as up-regulating adhesion molecules' expression, promoting intima proliferation and angiogenesis, and inducing oxidative stress. Cell and animal studies suggest that limiting RAGE expression in vascular cells could modulate expression of various pro-inflammatory mediators and prevent vascular dysfunction development [9].

PKC-beta has been recognized as a key mediator in AGEs-induced micro- and macro-vascular dysfunction [10]. Studies have demonstrated that AGEs can activate PKC-beta and induce a series of pathophysiological changes in cells [10]. In the present study, we demonstrated that selective blockade of PKC-beta with LY333531 can reverse AGEs' effect on macrophage adhesion to HUVEC and reduce AGEs-induced ECs damage.

Abbreviations: ECs, endothelial cells; TGF-beta1, transforming growth factor-beta1; OS, oxidative stress; ICAM-1, intercellular adhesion molecule-1; MCP-1, monocyte chemotactic protein-1.

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1567-5769/$ – see front matter.
doi:10.1016/j.intimp.2010.09.006

2. Materials and methods

2.1. Animals and reagents

All animal care and this investigation conform to the *Guide for the Care and Use of Laboratory Animals* published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996), and were approved by the Ethical Review Board of Macau University of Science and Technology and Consun Pharmaceutical Group. Male Swiss mice weighing 20–25 g were supplied by the Shanghai laboratory animal centre; food and water were given *ad libitum*.

The selective PKC-beta inhibitor, LY333531, was purchased from Alexis Biotechnologies (Nottingham, UK). Polyclonal ICAM-1 antibody was obtained from Boster (Wuhan, China); polyclonal TGF-beta1 and monoclonal RAGE antibodies were supplied by Santa Cruz Biotechnology (California, USA). The SOD and MDA detection kits were supplied by Jiancheng (Nanjing, China). All other reagents used were derived from commercial sources.

2.2. Preparation of advanced glycation end products

AGEs were prepared as previously described [11,12]. In general, BSA was added into 10 mM/L PBS (pH 7.4, concentration of 5 g/L), incubated with 50 mM/L D-glucose in 95% air/5% CO_2 at 37 °C for 12 weeks. Unincorporated glucose was removed by dialysis overnight against 0.01 M PBS. AGEs were stored at −20 °C until use.

2.3. Culture of Human Umbilical Vein Endothelial Cells

Human Umbilical Vein Endothelial Cell (HUVEC) line was purchased from ATCC (Manassas, VA, USA). Cells were cultured in DMEM (Gibco) supplemented with 10% FBS (fetal bovine serum), 1% penicillin–streptomycin, at 37 °C in a 95% air/5% CO_2 incubator.

2.4. Macrophages migration and adhesion assay

Macrophages were collected from mice abdomen according to the reports [13] with minor adjustment. In general, mice were executed by cervical dislocation, and peritoneal exudates were induced by one intraperitoneal injection of 20–25 ml sterile cold phosphate-buffer solution (PBS, pH 7.4). Ten minutes later, abdominal walls were shaved and cleansed with alcohol. Under sterile conditions, the peritoneal exudates were expelled by syringe and centrifuged at room temperature; after red blood cell quassation by 0.83% NH_4Cl, the solution was centrifuged at room temperature for two times and the macrophages were re-suspended by DMEM and adjusted to the density of 1×10^6.

To study macrophage migration (Fig. 1), HUVECs were firstly seeded on the coverslips in 24-well plates at confluence density of 80–90%. HUVECs were starved and incubated with BSA (200 μg/ml), AGEs (200 μg/ml) [12], or LY333531 (200 nM) [14] + AGEs (200 μg/ml) for 24 h. Then the 24-well plates were inserted with Transwell inserts (5.0 μm pore size polycarbonate membrane, Corning Costar Corporation, Cambridge, MA); the macrophages were plated into inserts and co-cultured with HUVEC for further 24 h. In this system [15], macrophages were grown on upper compartment of the Transwell microporous membranes. At 48 h, macrophages in the upper compartment were gently wiped with cotton to remove cells that didn't pass through the membrane, then the whole microporous membranes were stained with 10% crystal violet for 10 min; after washing with PBS, the upper side of the microporous membrane and the upper compartment walls were carefully wiped with cotton. Then the membranes were carefully clipped with razor blade (for microscopy observation) or washed with 30% glacial acetic acid (for macrophage counting determination). For macrophage counting determination, the washing solution was transferred to 96-well plate (Corning Costar) and detected at the wavelength of 580 nm.

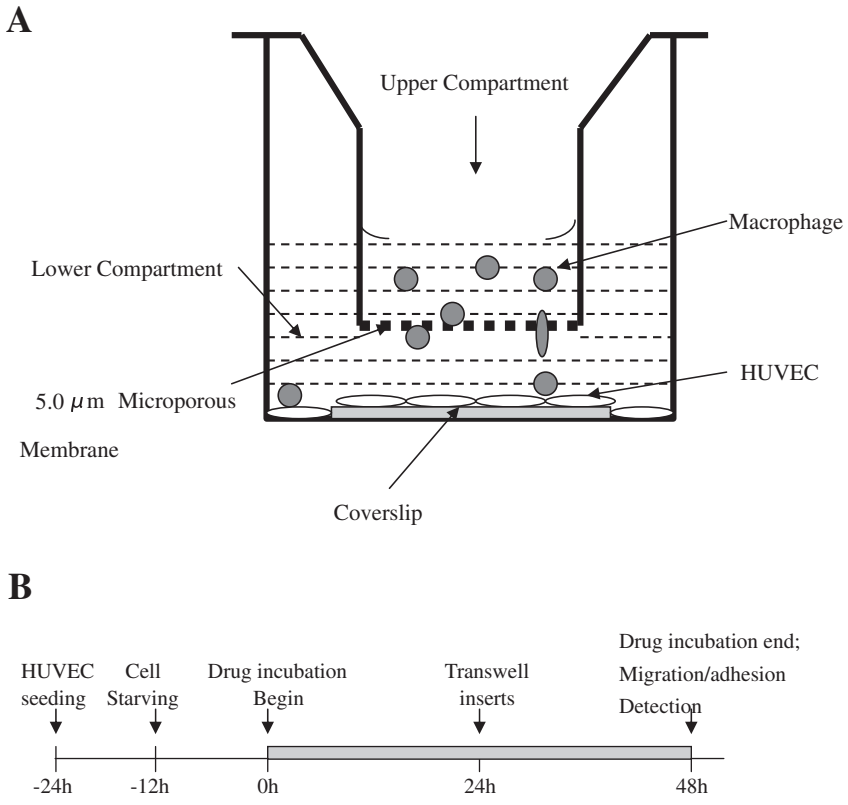

Fig. 1. Diagram of macrophage migration detection by Transwell. (A) Composition diagram of Transwell. (B) The detection flow of macrophage migration by Transwell. Following different treatment as described in Section 2.4, macrophage migration through microporous membranes was detected by 10% crystal violet staining or 30% glacial acetic acid washing.