Biochemical and Biophysical Research Communications 388 (2009) 67–72

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

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

Endothelial cells promote the proliferation of lymphocytes partly through the Wnt pathway via LEF-1

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ARTICLE INFO

Article history:
Received 19 July 2009
Available online 28 July 2009

Keywords:
LEF-1
Endothelial cell
Lymphocyte
Proliferation
Apoptosis

ABSTRACT

The function of T cells and B cells is to recognize specific "non-self" antigens, during a process known as antigen presentation. Once they have identified an invader, the cells generate specific responses that are tailored to maximally eliminate specific pathogens or pathogen-infected cells. Endothelial cells (ECs) can trigger the activation of T cells through their class I and class II MHC molecules. In this study, we examined the effect of ECs on the proliferation of lymphocytes. We report that the proliferation of T and B cells can be improved by interaction with ECs. LEF-1 is one of the main molecular mediators in this process, and the inhibition of LEF-1 induces apoptosis. These results suggest that LEF-1 modulates positively the proliferation of lymphocytes induced by their interaction with ECs.

Introduction

Endothelial cells (ECs) line the interior surface of blood vessels throughout the entire circulatory system, forming an interface between circulating blood in the lumen and the rest of the vessel wall. They not only keep cells within the blood from leaking out of the vessels, but also play an important role in the maintenance of the vessel wall [1–3]. Endothelial cells are heterogeneous [4,5] and can show different structural and functional characteristics in different tissues. As a barrier [6], the endothelium is semi-permeable and controls the transfer of small and large molecules. Endothelial cells are dynamic and are capable of conducting a variety of metabolic and synthetic functions [7,8]. These cells exert significant paracrine and endocrine actions through their influence on the underlying smooth muscle cells, or on circulating blood elements, such as platelets and white blood cells.

The most important recent development regarding endothelial cells concerns the knowledge of the cell surface molecules. These act as receptors and interaction sites for a host of important molecules, especially those that attract or repel lymphocytes. As part of their normal life cycle, many lymphocytes pass through the endothelial lining [9,10], especially in capillaries, so that they can monitor foreign agents (antigens) in tissues. Since lymphocyte recognition of, and response to foreign antigens occurs typically in lymphoid organs or in non-lymphoid tissue, the principles and mechanisms that regulate lymphocyte proliferation is critical to the generation of an immune response. Lymphocytes begin a process by which they rapidly reproduce themselves after they encounter an antigen or foreign protein, so that there are enough lymphocytes available that can recognize and fight the invading antigens. Although ECs have been reported to display class I and II MHC-peptide complexes were displayed on their surface and to activate lymphocytes [11], the role of the endothelium as the gatekeeper regulating lymphocyte interactions with tissues is more complex than for other cells. Lymphocytes not only adhere strongly to inflamed endothelium and play a critical role in the inflammatory response, but they also interact in a precisely regulated fashion with normal endothelium, and thereby migrate into lymphoid and non-lymphoid tissue [12,13]. Lymphocyte adhesion molecules are important in inflammation. While lymphocytes are normally repelled by the endothelium, to allow the free flow of blood cells over the surface, in inflammatory states they are attracted to the endothelium by adhesion molecules. They then pass in between endothelial cells by a process called diapedesis. During this process, lymphocytes can be activated by ECs [11]. It is not clear, however, whether the interaction between lymphocytes and ECs can induce the proliferation of lymphocytes, and what the molecular mechanism are.

The Wnt signaling pathway is one of a handful of evolutionarily-conserved signal transduction pathways used extensively during animal development, from Hydra to humans [14]. Activation of Wnt signal transduction pathways upon ligand binding can regulate diverse processes, including cell proliferation, migration, polarity, differentiation, and axon outgrowth [15]. In the

Abbreviations: ECs, endothelial cells; LEF-1, lymphoid enhancer factor 1; HGM, highly mobility group; TCF, T-cell factor

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0006-291X/$ - see front matter © 2009 Elsevier Inc. All rights reserved.
doi:10.1016/j.bbrc.2009.07.117

canonical pathway, Wnt binding leads to the stabilization of the transcription factor β-catenin, which enters the nucleus and interacts with lymphoid enhancer factor 1 (LEF-1) to regulate Wnt pathway target genes. LEF-1 is expressed in developing B and T cells, and at multiple sites of organogenesis during embryonic development [16]. LEF-1 has no transcriptional activation potential by itself, but it can act as an architectural protein in the assembly of multi-protein enhancer complexes, together with other lymphoid-specific proteins. In addition, LEF-1 is the most downstream factor in Wnt signaling. It interacts with β-catenin through amino-terminal sequences, and thus forms a ternary complex with DNA, mediating a transcriptional response to Wnt signaling. Several studies have shown that LEF-1 is one of the key regulators in the proliferation of lymphocytes [17,18].

In this study, we investigated the proliferation of lymphocytes induced by their interaction with ECs. We discovered that ECs can enhance the proliferation of lymphocytes. Furthermore, we showed that the expression of LEF-1 was increased gradually after the interaction with ECs. The absence of LEF-1 led to reduced proliferation and increased apoptosis of lymphocytes. Our results imply that LEF-1 might play an important role in the proliferation of lymphocytes induced by their interaction with ECs.

Materials and methods

EC culture. To culture ECs [19], mice were sacrificed by cervical dislocation and the aorta was dissected and cut coronally into segments under sterile conditions. The segments were washed in PBS on ice to remove blood, and were digested in collagenase IV/PBS for 1 h. Dispersed cells were washed again and cultured in 24-well plates with Dulbecco's modified Eagle's medium (DMEM) containing 20% FBS and endothelial cell growth supplement (ECGS) (BD Biosciences). Cells were characterized by FACS after staining with anti-105 and anti-VEGFR2 and cells between passages 2 and 5 were used in the experiments.

Proliferation. T or B cells were isolated from lymph nodes and sorted using anti-CD3 or anti-CD19 magnetic beads (Miltenyi Biotec GmbH, Germany) following the recommended protocol. Lymphocytes were incubated with carboxyfluorescein diacetate succinimidylester (CFSE, Sigma) at 37 °C for 10 min, and then in 5 ml of ice-cold Dulbecco's modified Eagle's medium (DMEM) for 5 min on ice. Cells were washed with cold DMEM medium and incubated with or without ECs (2×10^5) in 24-well plates. After 48 h, lymphocytes were collected and their proliferation was analyzed by FACS.

In some experiments, LEF-1 shRNA (m)-GFP Lentiviral Particles (and GFP Lentiviral Particles as control), purchased from Santa Cruz Biotechnology, Inc. (USA), were used following the manufacturer's instructions.

MTT assay. Lymphocytes (4×10^3 cells per well) were co-cultured with ECs (1×10^3) in 96-well plates, and T or B cells were collected on days 1–5 of the co-culture with 100 μl of the medium. An equal volume of fresh medium containing 20% MTT (5 mg/ml) was added. Cells were incubated further at 37 °C for 4 h, and then 150 μl of dimethyl sulfoxide (DMSO, Sigma) was added to each well, and mixed by shaking at room temperature for 10 min. The absorbance was then measured at 490 nm. Each experiment was repeated at least three times.

RT-PCR. Lymphocytes were disrupted in Trizol reagent (Invitrogen, Carlsbad, CA), and total cellular RNA was prepared according to the manufacturer's instructions. cDNA was prepared from the total RNA using a reverse-transcription kit (TOYOBO Co., Osaka, Japan). cDNA from equal amounts of total RNA was used to amplify target genes by PCR using Taq DNA polymerase (Takara Bio, Inc., Japan) with β-actin as a reference control. Primers used were as follows:

LEF-1F: 5′-TCCTTGGTGAACGAGTCTGAAA-3′;
LEF-1R: 5′-TCCTTGGTGAACGAGTCTGAAA-3′;
β-actin F: 5′-CATCCGTAAAGACCTCTATGCC AAC-3′;
β-actin R: 5′-ATGGAGCCACCGATCCACA-3′.

Western blotting. Whole-cell extracts were prepared by lysing cells with the RIPA buffer (50 mM Tris–HCl, pH 7.9, 150 mM NaCl, 0.5 mM EDTA, and 0.5% NP-40, 0.1 mM PMSF). Proteins were separated by 12% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE), and were electroblotted onto polyvinylidene difluoride membranes. Membranes were probed with rabbit-anti-mouse LEF-1 (Cell Signaling Technology, Danvers, MA), and monoclonal anti-β-actin (AC-74, Sigma, St. Louis, MI) at appropriate dilutions, followed by incubation with horseradish peroxidase-conjugated secondary anti-rabbit or anti-mouse IgG antibody (Sigma). Blots were developed using an enhanced chemiluminescence system (Roche, Basel, Switzerland).

Enzyme-linked immunosorbent assay. For the detection of cytokines, T and B cells were collected after co-cultured with ECs for2 - days. IL-4, IL-6, IL-10, IL-12, TNF-α, and INF-γ were analyzed by enzyme-linked immunosorbent assay (ELISA) kits (Jinmei Biotec, Shenzhen, China) following the recommended protocols.

Cell cycle analysis. Cells (1×10^6) were collected and washed with PBS, then fixed by incubating in 75% alcohol for 30 min at room temperature. The cells were washed three times with cold PBS, and resuspended in 1 ml PBS containing 40 μg propidium iodide (PI,Sigma) and 100 μg RNase A (Sigma), and incubated at 37 °C for 30 min. Samples were analyzed for DNA contents using a FACScalibur™ instrument (BD Immunocytometry Systems, San Jose, CA). Each experiment was repeated at least three times.

Apoptosis. Apoptotic cells were detected using the AnnexinV-FITC apoptosis Detection KIT I (Pharmingen, San Diego, CA), according to the manufacturer's instructions.

Statistics. Statistical analysis was performed with the SPSS 12.0 program. Results were expressed as means ± SD. Comparisons between groups were undertaken using Student's unpaired *t*-test. $P < 0.05$ was considered statistically significant.

Results

Endothelial cells can be obtained with our culture system

To examine the influence of endothelial cells on lymphocytes, we established an EC cultured system in vitro. Long segments of aorta were isolated to obtain relatively large amounts of ECs. The aortae were cut into small segments and were digested thoroughly with collagenase IV. After culturing for 3 days, the cells we seeded into 24-well plates became elongated and sharper. On day 5, the cells formed circles similar to a lumen structure (Fig. 1A). To identify other characteristics, we stained the cells with endothelial cell markers and found that they expressed high levels of CD105 and VEGFR2 (Fig. 1B). These results indicate that the cells we cultured were endothelial cells.

Co-culture with ECs enhanced the proliferation of T and B lymphocytes. Lymphocyte–endothelial cell interaction has been reported previously [20,21]. Therefore, we investigated the proliferation of T and B lymphocytes in vitro when they encountered ECs using the MTT assay. The growth of T or B cells was significantly slower than that of EC–T cells or EC–B cells (Fig. 2A). We further analyzed the cell cycle progression of lymphocytes and EC–lymphocytes. As shown in Fig. 2B, lymphocytes had a higher proportion of cells in the G_0/G_1 phase compared with that of EC–lymphocytes. We also examined the levels of cytokines that related to the proliferation of lymphocytes. In T cells, TNF-α, IFN-γ, and IL-12 were all at lower levels, compared with that in EC–T cells (Fig. 2C). B cells also in-