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CD40/CD40L interaction induces E-selectin dependent leukocyte adhesion to human endothelial cells and inhibits endothelial cell migration

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ABSTRACT

Background: CD40 is a receptor expressed on a wide range of cells such as leukocytes and endothelial cells (EC). As a member of the tumor necrosis factor (TNF) superfamily the activation of CD40 by CD40-ligand (CD40L) plays a crucial role for the development and progression of a variety of inflammatory processes including atherosclerosis. The aim of the present study was to investigate the effect of CD40/CD40L interaction on leukocyte adhesion to the endothelium and on endothelial cell migration.
Methods and results: Human umbilical vein endothelial cells (HUVEC) were stimulated with either stable transfectants of mouse myeloma cells expressing the CD40L or wild type cells (4 h). Subsequently adhesion of leukocytes expressing Sialyl Lewis X, the counterpart for E-selectin (HL60 cells), was measured under shear stress (2–2.6 dyne/cm^2) using a flow chamber adhesion assay. Stimulation of CD40 led to a significant increase of E-selectin dependent adhesion of leukocytes to the endothelium. Incubation of cells with either the CD40L blocking antibody TRAP-1 or the E-selectin blocking antibody BBA2 during CD40 stimulation completely abolished adhesion of leukocytes to HUVEC. Similar results were found in human cardiac microvasculature endothelial cells (HCMEC). In contrast stimulation of CD40 had no effect on adhesion of L-selectin expressing NALM6-L cells. Furthermore, CD40/CD40L interaction abrogated VEGF-induced migration of HUVEC compared to non-stimulated controls. In comparison experiments, stimulation of endothelial cells with VEGF led to a significant phosphorylation of ERK1/2, Akt, and eNOS. Stimulation of endothelial CD40 had no effect on VEGF-induced phosphorylation of ERK1/2. However, VEGF-induced activation of Akt and eNOS was reduced to baseline levels when endothelial CD40 was stimulated.
Conclusion: CD40/CD40L interaction induces E-selectin dependent adhesion of leukocytes to human endothelial cells and reduces endothelial cell migration by inhibiting the Akt/eNOS signaling pathway.

1. Introduction

CD40 is a member of the tumor necrosis factor (TNF) receptor superfamily, expressed on a broad range of cell types, including lymphocytes, macrophages, vascular smooth muscle cells, and endothelial cells [1,2]. Initially discovered as a part of the humoral immunity, it was later shown that CD40 plays a crucial role in a variety of pathophysiological states including inflammation, atherosclerosis, and angiogenesis [3,4]. While CD40 is expressed only at low levels on quiescent endothelial cells, it is upregulated in areas of inflammation [5]. CD40 interacts with its ligand CD40L (also known as CD154), expressed on activated CD4$^+$ T-cells, monocytes, and platelets [6–8]. CD40/CD40L ligation leads to increases in inflammatory cytokines, chemokines, matrix metalloproteinases, and adhesion molecules including selectins [8]. The family of selectins consists of three members: the leukocyte L-selectin and the vascular E- and P-selectin [9]. Selectins play a pivotal role for recruitment of leukocytes to sites of inflammation by enhancing leukocyte rolling [10].

Beside leukocyte adhesion, migration of endothelial cells is a key step in the progression of atherosclerosis. By using a scratched wound assay Urbich et al. [11] demonstrated that CD40/CD40L interaction plays an important role in VEGF-induced endothelial cell migration. It was shown that both the MEK/ERK signaling pathway and the Akt/eNOS signaling pathway are involved in CD40/CD40L-dependent endothelial cell migration [12–14]. Therefore the aim of the present study was first to investigate the impact of CD40/CD40L interaction on selectin-dependent leukocyte adhesion to the endothelium and second to examine the effect of CD40/CD40L interaction on endothelial cell migration and the underlying signaling mechanism.

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2. Materials and methods

2.1. Reagents and antibodies

The MEK-1 inhibitor PD98059 and antibodies against Akt, phospho-Akt (Ser^{473}), phospho-eNOS (Ser^{1177}), ERK1/2, and phospho-ERK1/2 (Thr^{202}/Tyr^{204}) were purchased from New England Biolabs. Antibodies against eNOS and PTEN were from Santa Cruz Biotechnology. Paramagnetic beads were from Dynal and α-FGF was from Boehringer. VEGF, antibiotics, HEPES, gelatin, collagenase II, bovine serum albumin, fibronectin, and UEA-I lectin were purchased from Sigma; Medium 199 Earle, L-glutamine, phosphate buffered saline, and fetal calf serum (FCS) were purchased from Biochrom. RPMI 1640 Medium and trypsin–EDTA were from Gibco BLR. Anti-E-selectin monoclonal antibody BBA2 was from Hermann Biermann GmbH, anti-CD40L monoclonal antibody TRAP-1 was kindly provided by Prof. Kroczek (Robert Koch Institute, Germany). Human TNF-α was from Pepro Tech, Hybond ECL nitrocellulose membrane, horseradish peroxidase linked anti-rabbit antibody, and ECL Western blotting detection reagents were from Amersham Life Sciences. Culture plastics were from Falcon.

2.2. Cell culture

For isolation of cardiac microvascular endothelial cells (HCMEC), human heart muscle segments (obtained from donor hearts) were enzymatically digested as previously described [15]. Cells were grown with Medium 199 containing fetal calf serum (20%), streptomycin (100 mg/mL), penicillin (100 U/mL), and endothelial cell growth factor (10 ng/mL) at 95% relative humidity and 5% CO_2 at 37 °C. Non-endothelial cells were removed from the cultures by treatment with Ulex europaeus-linked paramagnetic beads. Human umbilical vein endothelial cells (HUVEC) were harvested after dispase treatment and prepared as previously described [16]. Ethical approval was obtained from the local committee.

Three different cell species were selected on the basis of the surface expression of receptors or ligands required for selectin mediated adhesion and CD40 ligand expression.

Mouse myeloma cells P3x.WT and stable transfectants *P3x.TBA7* expressing CD40L were grown in suspension culture in RPMI-1640 with 10% fetal calf serum, and antibiotics in 5% CO_2 at 37 °C. Sufficient CD40L expression on P3x.TBA7 cells was assessed by flow cytometry.

HL60 cells with high expression of Sialyl Lewis X (sLex), the leukocyte counterpart for E-selectin, were grown under the same culture conditions as NALM-6 cells and exhibited high expression of sLex as measured by flow cytometry.

Human pre-B cells NALM6 and stable transfectants expressing L-selectin (*NALM6-L*) were grown in suspension culture in RPMI-1640 with 10% fetal calf serum, and antibiotics in 5% CO_2 at 37 °C; for NALM6-L geneticin (500 μg/mL) was added for a positive selection of the transfectants.

HL60, NALM6, and NALM6-L cells were a kind gift of Dr. A. Zakrzewicz, Department of Physiology, Free University Berlin, Germany. P3x.WT and P3x.TBA7 cells were kindly provided by Prof. Kroczek, Robert Koch Institute, Berlin, Germany.

2.3. Flow chamber adhesion assay

Endothelial cells were grown on fibronectin-coated coverslips and exposed to near physiologic flow conditions (2–2.6 dyne/cm^2) using a flow chamber as previously described [16]. In some experiments ECs were stimulated for 4 h with TNF-α (500 U/mL), P3x.TBA7 cells or P3x wildtype cells. In additional experiments, stimulated ECs were incubated with function blocking anti-E-selectin monoclonal antibody BBA2 (10 μg/mL). Usually 100 mL of cell suspension (HL60, NALM6 or NALM6-L cells) were added to the flowing perfusion medium and cell adhesion was allowed to take place during a 5 min flow. Afterward, the number of firmly attached cells was counted.

2.4. Migration assay

Migration of HUVEC was examined in transwell cell culture chambers as previously described [17]. Migration was induced by addition of VEGF (20 ng/mL) to the lower compartment. After a

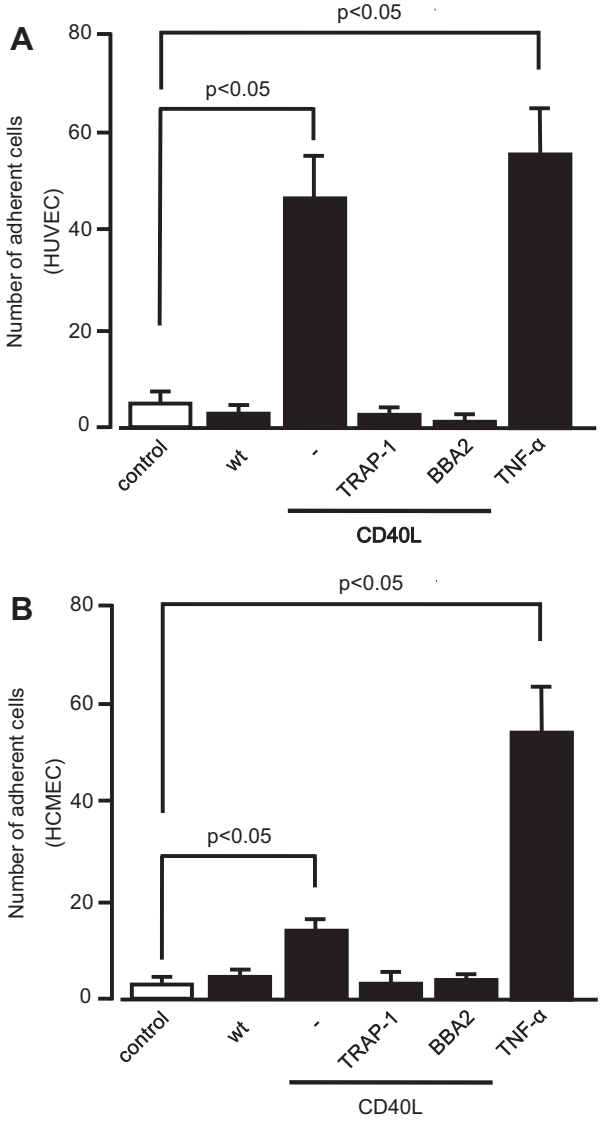

Fig. 1. Effect of CD40 stimulation on E-selectin dependent leukocyte adhesion to human endothelial cells. (A) Human vein endothelial cells (HUVEC) or (B) human coronary microvascular endothelial cells (HCMEC) were incubated under shear stress (2–2.6 dyne/cm^2) and stimulated with either wild-type myeloma cells P3x.WT (wt) or the stable transfectants P3x.TBA7 expressing CD40 ligand (CD40L) for 4 h. Subsequently 100 mL cell suspension of HL60 cells expressing sLex was added to the flowing perfusion medium. Adhesion of HL60 cells to endothelial cells was analyzed after 5 min of perfusion by counting of firmly attached cells. In additional experiments endothelial cells were incubated with the anti-CD40L moAB TRAP-1 (10 μg/mL) or with the anti-E-selectin moAb BBA2 (10 μg/mL) during CD40 stimulation period. For positive control cells were stimulated with 500 U/ml TNF-α for 4 h. Data are expressed as mean ± SE of at least $n = 5$ independent experiments.

5 h migration period, HUVECs migrated toward the lower membrane surface were fixed and stained using ice-cold methanol and Mayer's hemalaun. The number of HUVEC per high-power field (magnification ×320) that migrated after 5 h to the lower surface of the filters was determined microscopically (Axiovert 135 microscope, Zeiss). Four randomly chosen high-power fields were counted per filter.

2.5. Western blot analysis

Immunoblotting was performed with 10% reducing SDS–PAGE as previously described [18]. Semiquantitative densitometry was done with the National Institutes of Health program 1.62.

2.6. Statistics

Data are given as mean ± SE of n (3–5) separate experiments using independent cell preparations. The comparison of means between groups was performed by one way analysis of variance (ANOVA) followed by Bonferroni post hoc test. Changes of parameters within the same group were analyzed by multiple ANOVA analysis. Probability (P) values of less than 0.05 were considered significant.

3. Results

3.1. Role of CD40 stimulation for E-selectin dependent adhesion of leukocytes to endothelial cells

To investigate the impact of CD40/CD40L interaction on E-selectin dependent leukocyte adhesion to the endothelium, HUVECs, and HCMECs were stimulated with P3x.TBA7 cells stably transfected with CD40L for 4 h. Subsequently, adhesion of HL60 cells to ECs was compared under shear stress (2–2.6 dyne/cm^2). Fig. 1A shows that stimulation of HUVEC by CD40L led to a significant increase of firmly attached HL60 cells expressing sLex, the leukocyte counterpart for E-selectin, compared to non-stimulated controls. In contrast, stimulation of HUVEC with P3x wild type cells had no effect on leukocyte adhesion. Incubation of cells with the anti-CD40L moAB TRAP-1 during CD40 stimulation period completely abolished E-selectin dependent adhesion of leukocytes to human endothelial cells. Accordingly, incubation of cells with the anti-E-selectin moAB BBA2 during CD40L stimulation period abrogated the effect of CD40L on leukocyte adhesion. Stimulation of endothelial cells with TNF-α that is known to induce leukocyte adhesion to the endothelium served as a positive control [19]. As shown in Fig. 1B similar results were obtained for adhesion of HL60 cells to HCMEC. However, the effect of CD40L on leukocyte adhesion to HCMEC is much less distinctive than in HUVEC.

3.2. Impact of CD40 stimulation for L-selectin dependent adhesion of leukocytes to endothelial cells

To examine the role of L-selectin dependent adhesion of leukocytes to human microvascular endothelial cells, HCMEC were stimulated with CD40L expressing P3x.TBA7 cells for 4 h. Subsequently a cell suspension of wild-type human pre-B cells (NALM6) or stable transfectants expressing L-selectin (NALM6-L) was added to the flowing perfusion medium. Though stimulation of CD40 induced enhanced adhesion of NALM6 cells there was no further increase of NALM6-L cells expressing L-selectin (Fig. 2). In contrast, stimulation of human endothelial cells with TNF-α led only to a slight increase of NALM6 cells while there was a significant increase in adhesion of L-selectin expressing NALM6-L cells.

3.3. Effect of CD40 stimulation on endothelial cell migration

Next the effect of CD40 stimulation on EC migration was investigated. Therefore HUVEC were incubated with CD40L expressing P3x.TBA7 cells or wildtype P3x cells for 24 h. Afterward cell migration was induced by stimulation with VEGF. As shown in Fig. 3 VEGF-induced migration was completely abolished when CD40 was stimulated with P3x.TBA7 cells prior to migration. In contrast

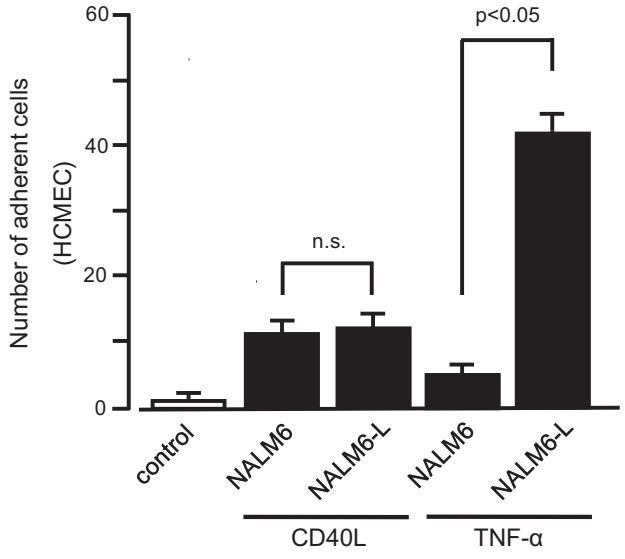

Fig. 2. Adhesion of L-selectin expressing pre-B cells to human coronary microvascular endothelial cells (HCMEC) stimulated with CD40L. HCMEC incubated under shear stress (2–2.6 dyne/cm^2) were stimulated with either the stable transfectants P3x.TBA7 expressing the CD40 ligand (CD40L) or with 500 U/mL TNF-α for 4 h. Subsequently 100 mL cell suspension of wild-type human pre-B cells (NALM6) or of stable transfectants expressing L-selectin (NALM6-L) was added to the flowing perfusion medium. Adhesion of cells to HCMEC was analyzed after 5 min of perfusion. Data are expressed as mean ± SE of at least n = 5 independent experiments, n.s. = no significant difference.

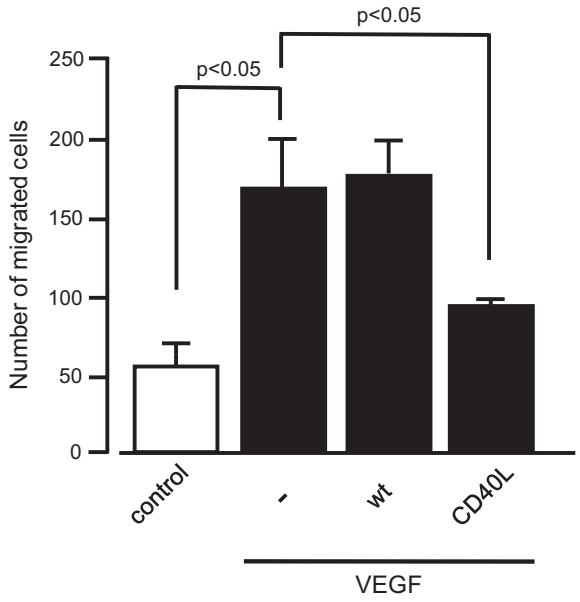

Fig. 3. Effect of CD40 stimulation on VEGF-induced endothelial cell migration. HUVEC were placed into the upper compartment of a transwell cell culture chamber. VEGF (20 ng/mL) was added to the lower compartment. After 5 h, migrated endothelial cells were fixed and counted. In part cells were pre-incubated with either wild-type myeloma cells P3x.WT (wt) or with the stable transfectants P3x.TBA7 expressing the CD40 ligand (CD40L) for 24 h. Data are expressed as mean ± SE of at least n = 5 independent experiments.

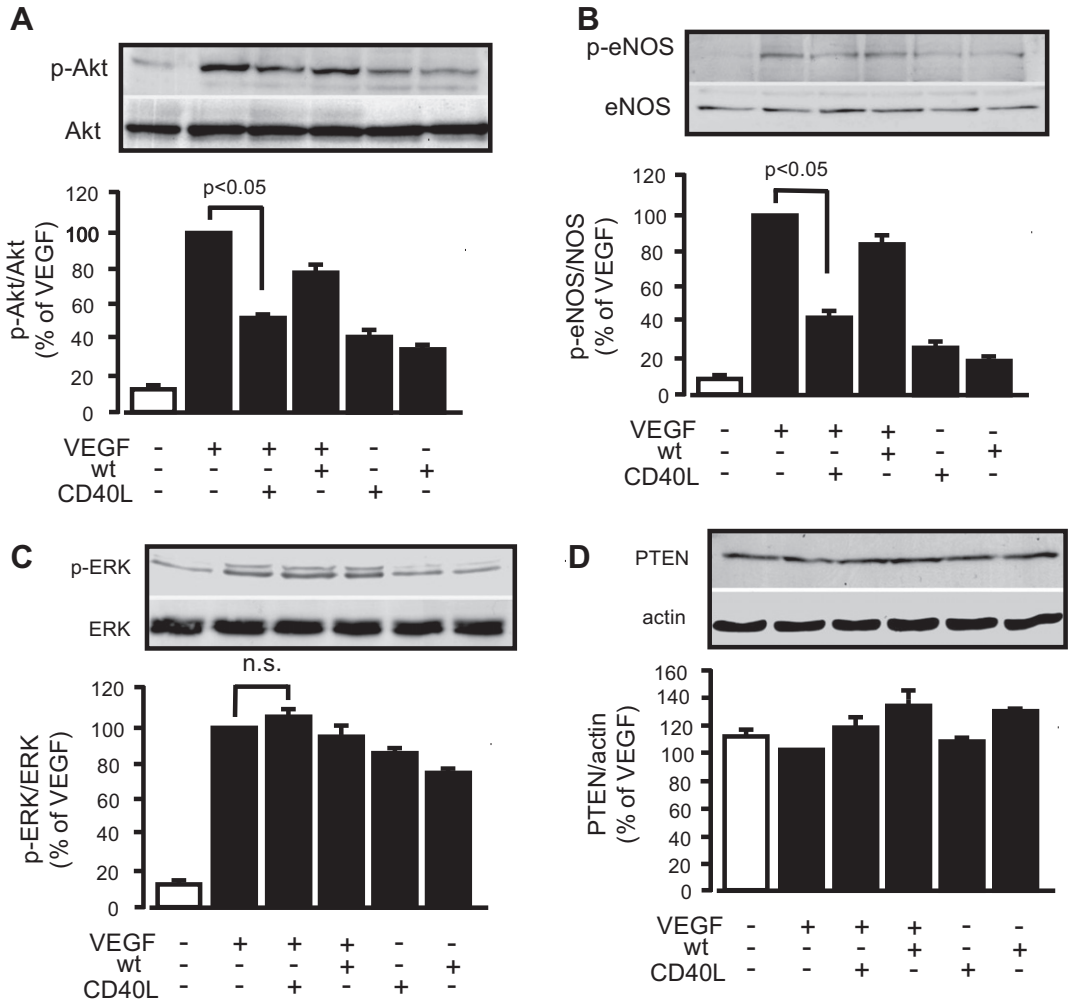

Fig. 4. Effect of CD40 stimulation on VEGF-induced p-ERK, p-Akt, p-eNOS, and PTEN. Human vein endothelial cells (HUVEC) were stimulated with either wild-type myeloma cells P3x.WT (wt) or with the stable transfectants P3x.TBA7 expressing the CD40 ligand (CD40L) for 4 h. In part HUVEC were incubated with VEGF (20 ng/mL) for 30 min after stimulation period. Subsequently phosphorylation of (A) Akt, (B) eNOS, (C) ERK 1/2, and (D) expression of PTEN was analyzed by Western blot. Bar graphs represent the densitometric analysis of phosphorylated protein relative to total protein for Akt, eNOS, and PTEN expression relative to actin. The ratio of VEGF was set to 100. Data are expressed as mean ± SE of at least $n = 3$ independent experiments, n.s. = no significant difference.

stimulation with wild type cells had no effect on VEGF-induced migration of human endothelial cells.

We and others have demonstrated that VEGF-induced migration of endothelial cells is regulated by the Akt/eNOS pathway as well as by the MEK/ERK pathway [12–14]. We therefore tested the effect of CD40 stimulation on VEGF-induced activation of these signaling pathways. Fig. 4 shows that 30 min incubation of human endothelial cells with VEGF led to a significant increase of Akt (Ser^{473}), eNOS (Ser^{1177}) and ERK 1/2 (Thr^{202}/Tyr^{204}) phosphorylation. When endothelial cells were pre-incubated with CD40L expressing P3x.TBA7 cells, VEGF-induced phosphorylation of Akt, and eNOS was significantly reduced. However, CD40 stimulation had no effect on VEGF-induced ERK1/2 signaling. According to VEGF-induced migration, mock treatment with P3x wild type cells had no effect on Akt or eNOS phosphorylation. As shown in Fig. 4D neither the stimulation with VEGF alone nor the pre-incubation of endothelial cells with CD40L expressing P3x.TBA7 cells or with P3x wild type cells had an effect on PTEN expression, the inhibitor of PI3-Kinase.

4. Discussion

In the present study we demonstrate that leukocyte adhesion to human endothelial cells crucially depends on CD40/CD40L interaction. We found that enhanced leukocyte adhesion following CD40 stimulation is mediated by an E-selectin dependent mechanism, while L-selectin is not involved. In addition, we showed that EC migration is inhibited by CD40/CD40L interaction. This inhibitory effect of CD40 stimulation is accompanied by decreases of Akt/eNOS signaling while ERK1/2 phosphorylation is not affected.

Leukocyte adhesion to endothelial cells is a multistep process that is initialized by the adhesion molecules E-, P- and L-selectin, and their corresponding ligands, e.g. sLex and PSGL-1 [20,21]. While L-selectin is constitutively expressed on leukocytes, E-selectin is found on activated endothelial cells. We could show that stimulation of endothelial CD40 enhances adhesion of leukocytes expressing sLex, the leukocyte counterpart for E-selectin, to human endothelial cells. This effect was found in both HUVEC and HCMEC. However, adhesion to endothelial cells isolated from human cardiac microvasculature was much weaker as adhesion to HUVEC. This fact might be due to different levels of E-selectin expression following treatment with pro-inflammatory cytokines like TNF-α in endothelial cells derived from different origin [22,23]. Enhanced leukocyte adhesion following CD40 stimulation could be prevented by using both a specific antibody blocking CD40L and by using an E-selectin blocking antibody. These results are in line with findings from Hollenbaugh et al. [2] demonstrating an upregulation of

E-selectin and adhesion of leukocytes to human endothelial cells in response to stimulation with recombinant CD40L. Accordingly Omari et al. [24] showed increased levels of E-selectin and adhesion of T-cells to human brain endothelial cells following CD40 stimulation with soluble CD40L.

According to Zakrzewicz et al. [25] we showed that TNF-α induces L-selectin dependent leukocyte adhesion to HCMEC. Even though CD40 stimulation of HCMEC enhanced adhesion of wild type human pre-B cells (NALM6), we found no further increase in adhesion of human pre-B cells that were stably transfected with L-selectin (NALM6-L), indicating that CD40/CD40L interaction does not affect L-selectin dependent leukocyte adhesion to HCMEC.

We further investigated the effect of CD40/CD40L interaction on EC migration, as there are several reports with contrary results. While Deregibus et al. [13] and Mach et al. [26] showed that CD40 stimulation induces in vitro angiogenesis and tube formation, Urbich et al. [11] demonstrated that CD40/CD40L interaction significantly reduces VEGF-induced EC migration. To further resolve this matter we used an assay that directly measures the number of migrated endothelial cells in response to VEGF stimulation. According to Urbich et al. [11] we found that CD40/CD40L interaction significantly reduces VEGF-induced EC migration. In line with previous results we found that VEGF induces activation of ERK1/2, Akt, and eNOS [15,27]. While CD40 stimulation had no effect on VEGF-induced ERK1/2-phosphorylation, the activation of Akt and eNOS was significantly reduced by CD40/CD40L interaction. These results are in agreement with findings of Tai et al. [28] emphasizing the relationship between CD40 and PI3K/Akt signaling. PTEN, a negative regulator of PI3-Kinase, was neither altered after VEGF stimulation nor did CD40 stimulation have any effect. Similar results were reported by Huang et al. [29] who showed that expression of PTEN remained unaltered while phosphorylation of Akt was enhanced by VEGF.

In summary the data of the present study point out the crucial role of CD40/CD40L interaction for the regulation of key elements of atherosclerosis. Firstly, CD40/CD40L interaction induces E-selectin dependent recruitment of leukocytes to the endothelium and secondly reduces EC migration by inhibiting Akt/eNOS signaling. These results emphasize the impact of targeting CD40/CD40L interaction in the treatment of atherosclerosis.

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