SUPPLEMENT MATERIAL

Inflammatory interaction between LIGHT and Proteinase-activated receptor-2 in endothelial cells – potential role in atherogenesis

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Materials and Methods

Isolation of cells

Peripheral blood mononuclear cells (PBMC) were obtained from heparinized blood by Isopaque-Ficoll (Lymphoprep; Nycomed, Oslo, Norway) gradient centrifugation. Further separation of monocytes (anti-human CD14-labeled magnetic beads; MACS, Miltenyi Biotec, Bergisch Gladbach, Germany) and CD3+ T cells (negative selection by monodisperse immunomagnetic beads; Dynal, Oslo, Norway) was performed as described elsewhere.1,2 Selected T cells consisted of >90% CD3+ cells and the isolated monocytes of >95% CD14+ cells (flow cytometry). The cells were stored in liquid nitrogen as pellets for RNA isolation or used for further in vitro experiments (monocytes).

Cell culture experiments

*Human umbilical vein endothelial cells* (HUVEC) were obtained from umbilical cord veins by digestion with 0.1% collagenase A (Roche Diagnostics GmbH, Mannheim, Germany)3 and cultured as previously described.4 HUVEC were passaged by treatment with 0.05% trypsin-EDTA (Gibco, Grand Island, NY) and grown in 12-well plates (Costar, Cambridge, MA) to confluence for 3 to 5 days. The medium was then discarded, and HUVEC were stimulated with different concentrations of recombinant human
(rh)LIGHT (R&D Systems, Minneapolis, MN), the PAR-2 agonist peptide sequence SLIGKV (a kind gift from Ola R Blingsmo at the Biotechnology Centre of Oslo, Oslo, Norway), or a combination thereof. In some experiments, 10-100 µM SP600125 (anthra[1,9-cd]pyrazol-6(2H)-one) (Sigma, St Louis, MO), 10 µM nuclear factor-kB (NF-kB) activation inhibitor IV (Calbiochem, San Diego, CA), or 20 µM NF-κB activation inhibitor II, JSH-23 (Calbiochem) was added to HUVEC culture 30 minutes before rhLIGHT stimulation. The cells used in the HUVEC experiments were in passage 3-5. The human monocytic cell line THP-1 (American Type Culture Collection, Rockville, MD) and freshly isolated human monocytes were cultured for 4 days in RPMI 1640 (PAA laboratories, Pasching, Austria) supplemented with 2.5% fetal bovine serum (Gibco) in the presence of rhTNFα (5 ng/ml, R&D Systems), before further incubation with or without rhLIGHT (100 ng/ml). At different time points, cell-free supernatants and cell pellets of HUVEC and monocytes were harvested and stored at -80°C. The endotoxin levels of all stimulants and culture media were <10 pg/mL (Limulus Amebocyte Assay; BioWhittaker, Walkersville, MD). In all experiments with controls and un-stimulated cells, the vehicle of the stimulus was always added.

Microarray

RNA was isolated from HUVEC using RNeasy (Quiagen, Hilden, Germany). Three µg of total RNA was used to generate cRNA, subsequently hybridized to Genechip Human Genome U133A Array (Affymetrix, Santa Clara, CA) according to standard protocols (http://www.affymetrix.com/support/technical/manual/expression_manual.affx). Arrays were scanned using a GeneArray scanner (Agilent Technologies, Palo Alto, CA) with
probe intensities extracted, and subjected to global scaling and statistical evaluations using the free software R.

**Real time quantitative RT-PCR**

Total RNA was extracted from HUVEC, monocytes, and T cells using RNeasy columns (Qiagen), subjected to DNase I treatment, and stored in RNA storage solution (Ambion, Austin, TX) at −80°C. In the mice experiments, tissue samples were cryohomogenized with a Mikro Dismembrator S (B Braun Biotech International GmbH, Melsungen, Germany), and total RNA was isolated with the RNeasy extraction kit (Qiagen), and reverse transcribed to cDNA using random hexamers and Superscript II reverse transcriptase (Life Technologies, Rockville, MD). Quantification of mRNA was performed using ABI Prism 7500 Fast Realtime PCR System (Applied Biosystems, Foster City, CA). Primers were designed using the Primer Express software, version 2.0 (Applied Biosystems, Foster City, CA). Primer sequences could be provided by request. PCR was performed using qPCR Master Mix for SyBr Green I (Eurogentec, Seraing, Belgium) and 300 nM primers. The mRNA levels of the housekeeping genes β-actin and 18S were used for normalization.

**Western blotting**

Western blotting was performed as previously described, separating equal amounts of protein from each sample by SDS-PAGE (10%) before transferring it onto polyvinyl difluoride membranes (NEN; Life Science, Boston, MA). The membranes were incubated with rabbit antibody against PAR-2 (Phoenix Pharmaceuticals, Belmont, CA), stripped, and reprobed with human anti-β-tubulin (Sigma) to ensure equal loading, followed by incubation with species-specific horseradish peroxidase-coupled secondary antibodies.
(Cell Signaling, Beverly, MA). In a subset of experiments, the siRNA-mediated gene silencing efficiency (see below) was examined using primary antibody against HVEM (goat; Santa Cruz Biotechnology, Santa Cruz, CA) and LTβR (mouse; R&D Systems). The immune complex was visualized by using the Supersignal West Pico Western blot detection system (Pierce, Rockford, IL), exposure to Hyperfilm ECL (Amersham Biosciences, Buckinghamshire, UK), and detected with the use of the Kodak 440 CF imaging station (Boston, MA). The software Total Laboratory v.1 10 (Phoretix, Newcastle, UK) was used for quantification.

**Preparation and transfection of siRNA**

Pre-designed siRNA against HVEM (siRNA ID: 13868) and LTβR (siRNA ID: 143461) were purchased from Ambion, and certificated non-silencing scramble control siRNA (cat No: 1022076) was obtained from Qiagen. HUVEC were cultured in 12-wells trays (Costar) and at 80% confluence, the cells were transfected using HiPerFect reagent (Qiagen) and 50 nM siRNA. The transfection reagents and the siRNA duplexes were mixed by vortexing in OptiMEM (Invitrogen), and incubated for 10 minutes at room temperature before the complexes were added drop-wise onto the cells. Thereafter, the cells were incubated for 18 hours with transfection complexes in normal growth medium. After an additional 18 hours, the cells were washed once and cultured for up to 8 hours with or without rhLIGHT (100 ng/ml) before harvesting and subsequent real time RT-PCR analyses. A parallel tray of siRNA-treated cells were assessed for HVEM and LTβR by Western blot to ensure successful gene silencing. The toxicity after siRNA transfections was examined for lactate dehydrogenase leakage using a cytotoxicity detection kit (Roche Applied Science, Penzberg, Germany).
Multiplex

Protein extracts (15 μg) from HUVEC, stimulated with rhLIGHT (100 ng/ml) or vehicle for 15 and 120 minutes, were subjected to phosphoprotein detection array (multiplexable beads: phosphorylated [p]-Akt; p-activating transcription factor-2 [ATF-2]; p-extracellular signal-regulated kinase [ERK]1/2; p-inhibitory [I]κB-α; p-Jun N-terminal kinase [JNK]; p-p38 mitogen-activated protein kinase [MAPK]; p-signal transducer and activator of transcription [STAT]3; BioRad, Hercules, CA) using the Bioplex (BioRad) suspension array technology. Multiplex suspension technology was also used for examining chemokine levels in supernatants of THP-1 cells and HUVEC (Biosource Human Chemokine Plex, Biosource, Camarillo, CA) analyzing concentrations of MCP-1, interferon (IFN)-γ-induced protein (IP)-10, monokine induced by IFN-γ (MIG), and regulated on activation normally T-cell expressed and secreted (RANTES). In all experiments, the samples were analyzed on a Multiplex Analyser (BioRad), and quantification was accomplished by using the BioPlex Manager Software (BioRad).

Enzyme immunoassays (EIAs)

Levels of macrophage chemoattractant protein (MCP)-1, interleukin (IL)-6, IL-8, IL-10, TNFα, and LIGHT were measured by EIAs (R&D Systems).

Mice

Female ApoE−/− and wild-type C57BL/6 mice were obtained from Taconic Europe A/S (Lille Skensved, Denmark) and fed standard chow and water ad libitum as previously described.7 Eight ApoE−/− and 8 C57BL/6 mice were euthanized at 18 weeks of age, and the aortas were freed from connective tissue under microscope and snap-frozen.

Immunohistochemistry
Acetone-fixed sections of the ascending aorta of the ApoE−/− and wild-type C57BL/6 mice were further fixed in formalin and stained using affinity-purified polyclonal goat anti-human LIGHT, HVEM, LTβR, or PAR-2 IgG (all Santa Cruz Biotechnology), rat anti-mouse CD68 IgG (macrophage marker; Serotec Ltd., Oxford, UK), or sheep anti-rat von Willebrand factor (vWF) IgG (endothelial cell marker; Cedarlane, Ontario, Canada). The primary antibodies were followed by biotinylated anti-goat, anti-rat or anti-sheep IgG (Vector Laboratories, Burlingame, CA). The immunoreactivities were further amplified using avidin-biotin-peroxidase complexes (Vectastain Elite kit, Vector Laboratories). Diaminobenzidine was used as the chromogen in a commercial metal enhanced system (Pierce). The sections were counterstained with hematoxylin. Omission of the primary antibody served as a negative control.

**Patients and controls**

In a separate experiment, we analyzed PAR-2 expression in monocytes and T cells from 14 patients with unstable angina (60±12 years, 6 women and 8 men), 14 patients with stable angina (66±8 years, 2 women and 12 men), and 10 healthy controls (59±6 years, 2 women and 8 men). Serum levels of C-reactive protein (CRP) were significantly higher in angina patients (p<0.01) as compared with controls (1.0±0.2 mg/l) with particularly high levels in those with unstable disease (2.2±0.5 mg/l versus 3.5±0.7 mg/l, stable and unstable angina, respectively; p<0.05). All patients with unstable angina had experienced ischemic chest pain at rest within the preceding 48 hours (ie, Braunwald’s class IIIB), but with no evidence of myocardial necrosis by enzymatic criteria. Transient ST-T segment depression and/or T-wave inversion were present in all cases. All patients with stable
angina had stable effort angina of >6 months duration and a positive exercise test. The diagnosis of coronary artery disease (CAD) was confirmed in all patients by coronary angiography showing at least 1 vessel disease (>50% narrowing of luminal diameter). Exclusion criteria were myocardial infarction or thrombolytic therapy in the previous month, ECG abnormalities invalidating ST-segment analyses, concomitant inflammatory diseases such as infections and autoimmune disorders, and liver or kidney disease.

**Ethics**

In the studies involving humans, informed consent for participation was obtained from all individuals. The studies were conducted according to the ethical guidelines at our hospital according to the Declaration of Helsinki and were approved by the local ethical committee. All animal experiments were in accordance with national guidelines and approved by the local ethical committee.

**Statistical analysis**

For statistical analysis of the *in vitro* experiments in HUVEC and monocytes, the Student paired *t* test was used. For comparisons of three groups of individuals, the nonparametric Kruskal-Wallis test was used. If a significant difference was found, Mann-Whitney *U* test was used to calculate the difference between each pair of groups. For comparisons within the same individuals, Wilcoxon’s matched-pair test was used. Probability values (2-sided) were considered significant at a value of <0.05.
References for Supplemental Material


Supplemental Figure Legends

Supplemental Figure I. The effect of PAR-2/LIGHT co-stimulation on PAI-1 and eNOS expression in HUVEC. Cells were incubated with the PAR-2 activating peptide SLIGKV (100 µM), rhLIGHT (100 ng/ml), or a combination thereof for 5 hours before analyzing mRNA levels of PAI-1 (A) and eNOS (B) by means of real-time RT-PCR and normalized to β-actin expression. **p<0.01 versus un-stimulated (unstim) cells; #p<0.05 versus SLIGKV (100 µM) when given alone. LIGHT/SLIGKV versus LIGHT (100 ng/ml) alone, p=0.08. The data are mean±SEM (n=6).

Supplemental Figure II. LIGHT-mediated JNK activation is mediated through HVEM. Silencing of HVEM, but not of LTβR, down-regulated the LIGHT-mediated (100 ng/ml) activation of JNK as assessed by attenuated phosphorylation of JNK (p-JNK) 15 minutes after LIGHT activation (phospho-protein multiplex suspension array technology). *p<0.05 versus scramble control. The data are mean±SEM (n=4).

Supplemental Figure III. Co-stimulation with LIGHT and PAR-2 agonist enhances PAR-2 expression. THP-1 monocytes (A) and HUVEC (B) were incubated with the PAR-2 activating peptide SLIGKV (100 µM), rhLIGHT (100 ng/ml), or a combination thereof for 5 hours before analyzing mRNA levels of PAR-2 by means of real-time RT-PCR and normalized to β-actin expression. **p<0.01 versus un-stimulated (unstim) cells; #p<0.05 versus SLIGKV (100 µM) and LIGHT (100 ng/ml) when given alone. The data are mean±SEM (n=8).
Supplemental Figure I

A  

PAI-1 : β-actin

B  
eNOS : β-actin

unstim  LIGHT  SLGKV  LIGHT/SLGKV

**  **  #  **  **  **
Supplemental Figure II

![Bar graph showing LIGHT stimulated P-JNK (% of Scambled)](chart)

- Scrambled
- siHVEM
- siLTβR

Significant difference indicated by *
Supplemental Figure III

![Bar chart A](image)

A

![Bar chart B](image)

B

**A**

**PAR-2 : β-actin**

<table>
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**B**

**PAR-2 : β-actin**

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**Significance:**

- ****: p < 0.05
- ****: p < 0.01
- ****: p < 0.001
- ****: p < 0.0001

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