Atherosclerosis 212 (2010) 451–456

Contents lists available at ScienceDirect

Atherosclerosis

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

Receptor for advanced glycation endproducts mediates pro-atherogenic responses to periodontal infection in vascular endothelial cells

Andreas Pollreisz[a,b], Barry I. Hudson[b], Jong S. Chang[b], Wu Qu[b], Bin Cheng[c], Panos N. Papapanou[a], Ann Marie Schmidt[b], Evanthia Lalla[a,*]

[a] Division of Periodontics, College of Dental Medicine, Columbia University, 630 W. 168th Street, PH7E-110, New York, NY, United States
[b] Division of Surgical Science, College of Physicians & Surgeons, New York, NY, United States
[c] Department of Biostatistics, Mailman School of Public Health, Columbia University, New York, NY, United States

ARTICLE INFO

Article history:
Received 10 December 2009
Received in revised form 28 June 2010
Accepted 10 July 2010
Available online 21 July 2010

Keywords:
RAGE
Infection
P. gingivalis
Periodontitis
Endothelial cells
Vascular inflammation
Atherosclerosis

ABSTRACT

Objective: A link between periodontal infections and an increased risk for vascular disease has been demonstrated. *Porphyromonas gingivalis*, a major periodontal pathogen, localizes in human atherosclerotic plaques, accelerates atherosclerosis in animal models and modulates vascular cell function. The receptor for advanced glycation endproducts (RAGE) regulates vascular inflammation and atherogenesis. We hypothesized that RAGE is involved in *P. gingivalis*'s contribution to pro-atherogenic responses in vascular endothelial cells.

Methods and results: Murine aortic endothelial cells (MAEC) were isolated from wild-type C57BL/6 or RAGE−/− mice and were infected with *P. gingivalis* strain 381. *P. gingivalis* 381 infection significantly enhanced expression of RAGE in wild-type MAEC. Levels of pro-atherogenic advanced glycation endproducts (AGEs) and monocyte chemoattractant protein 1 (MCP-1) were significantly increased in wild-type MAEC following *P. gingivalis* 381 infection, but were unaffected in MAEC from RAGE−/− mice or in MAEC infected with DPG3, a fimbriae-deficient mutant of *P. gingivalis* 381. Consistent with a role for oxidative stress and an AGE-dependent activation of RAGE in this setting, both antioxidant treatment and AGE blockade significantly suppressed RAGE gene expression and RAGE and MCP-1 protein levels in *P. gingivalis* 381-infected human aortic endothelial cells (HAEC).

Conclusion: The present findings implicate for the first time the AGE-RAGE axis in the amplification of pro-atherogenic responses triggered by *P. gingivalis* in vascular endothelial cells.

1. Introduction

The relationship between infection, inflammation and atherosclerosis has been highlighted in recent years [1]. Periodontal diseases are chronic infections affecting the supporting structures of teeth with a high prevalence in most populations [2]. A link between periodontal infections and atherosclerosis-related vascular disease has been demonstrated [3]. The major periodontal pathogen, *Porphyromonas gingivalis*, has been shown to gain access to the systemic circulation, accelerate atherosclerosis in animal model studies, invade vascular cells and induce pro-atherogenic and procoagulant responses [4–11].

In addition to having important roles in body homeostasis, endothelial cells (ECs) serve as regulators of lipid transport and transduce inflammatory signals. The ability of *P. gingivalis* to interact with ECs is of importance, as EC injury leading to dysfunction is crucially involved in vascular inflammation and atherogenesis [1].

The multi-ligand receptor for advanced glycation endproducts (RAGE) is expressed in multiple cell types relevant to atherogenesis, including ECs, and its genetic deletion in atherosclerosis-prone mice has been shown to suppress vascular injury and atherosclerotic plaque formation [12]. Hyperglycemia, inflammation and oxidative stress drive the generation of RAGE and its ligands, such as advanced glycation endproducts (AGEs), high-mobility group box 1 (HMGB1) and S100/calgranulins, and RAGE confers its impact and that of its ligands via signal transduction pathways [13]. Previous studies have revealed a role for RAGE and its ligands in the pathogenesis of diabetes-associated periodontitis via amplification of the inflammatory response to the bacterial challenge and delayed bone healing [14,15].

Our present studies aim to explore mechanisms underlying accelerated atherogenesis upon *P. gingivalis* infection and are based on the hypothesis that RAGE is involved in the pro-atherogenic responses and endothelial activation elicited by this periodontal pathogen.

* Corresponding author. Tel.: +1 212 305 9283; fax: +1 212 305 9313.
E-mail address: EL94@columbia.edu (E. Lalla).

0021-9150/$ – see front matter © 2010 Elsevier Ireland Ltd. All rights reserved.
doi:10.1016/j.atherosclerosis.2010.07.011

2. Methods

2.1. Animals, cells and bacterial strains

All procedures were approved by the Institutional Animal Care and Use Committee of Columbia University. Mice were housed in a pathogen-free environment and allowed free access to normal rodent chow and water. Male C57BL/6 were purchased from Jackson Laboratories (Main Harbor, ME). Homozygous RAGE−/− mice were backcrossed for more than 12 generations into C57BL/6 background. Genomic DNA was isolated from tail biopsies and PCR was used to identify the deficiency of the RAGE gene. Mouse aortic endothelial cells (MAEC) were isolated from 6- to 8-week old C57BL/6 or RAGE−/− mice, and maintained in DMEM/F12HAM medium (Gibco, Grand Island, NY) with 10% fetal bovine serum (Thermo Scientific HyClone, Logan, UT) as previously described [16]. Cells were serum-starved overnight prior to infection and were maintained in serum-free medium after infection. Primary human aortic endothelial cells (HAEC, Lonza, San Diego, CA) were maintained in endothelial cell medium 2 (EGM-2, Lonza) with a serum concentration of 2% and other supplements provided by the supplier. *P. gingivalis* FDC381 and DPG3, its fimbriae-deficient mutant, were grown in anaerobic chambers at 37 °C on blood agar plates without and with erythromycin, respectively (Anaerobe Systems, Morgan Hill, CA). Bacterial suspensions were prepared in phosphate buffered saline without Mg^{2+}/Ca^{2+} (PBS) using established growth curves and spectrophotometric analysis.

2.2. Infection of aortic endothelial cells with P. gingivalis

MAEC or HAEC were plated (1.5×10^5/well) in 6-well plates (Corning, Acton, MA) for 24 h, and infected in antibiotic-free medium with *P. gingivalis* 381 or DPG3 for 90 min (multiplicity of infection [MOI] of 1:100, calculated based on the number of cells per well when seeded), or left uninfected. Cells were then washed with PBS and maintained for 6 or 24 h. Supernatants and cell lysates (prepared using M-PER reagent, Thermo Scientific, Waltham, MA) were stored at −70 °C until further analysis.

2.3. AGE and MCP-1 ELISA

To measure AGE levels in cell supernatants, an enzyme linked immunosorbent assay (ELISA) was performed as previously described [17]. Briefly, samples were first incubated with blocking buffer (5% GSA, 1% BSA in 0.1 M PBS, pH 7.4) for 1 h at room temperature and then an affinity-purified chicken anti-AGE antibody was used for 3 h (Thermo Scientific, 1:100 in blocking buffer). The secondary antibody (anti-chicken IgG, Sigma) was diluted 1:10,000 and used for 1 h at room temperature. Signals were developed and read at 490 nm. Ribose glycated albumin was used to prepare the standard curve. Results are given in AGE units, established elsewhere (calculated as ng AGE-BSA/mg soluble protein) [18]. Levels of mouse and human MCP-1 were determined in cell culture supernatants by commercially available ELISA kits (Bender MedSystems, Burlingame, CA) according to the manufacturer's instructions. In blocking experiments, HAEC were preincubated with anti-RAGE IgG (70 μg/mL), or anti-AGE IgG (15 μg/mL), or aminoguanidine (Sigma, 200 μmol/L) for 2 h [19], or with *N*-acetyl-L-cysteine (NAC, Sigma, 10 mmol/L), or diphenylene iodonium (DPI, Sigma, 30 μmol/L) for 1 h and then infected with *P. gingivalis* 381 for 90 min. Aminoguanidine is an established AGE formation inhibitor, NAC is a well-characterized thiol-containing antioxidant and DPI is an inhibitor of flavine-binding proteins such as NADPH oxidase, the primary source of reactive oxygen species (ROS) in ECs. Non-specific IgG antibody (Alpha Diagnostic, San Antonio, TX, 15 μg/mL) was used as a negative control. Following the PBS wash, blocking agents were reintroduced until the 6 h time point when supernatants were collected for MCP-1 level assessments.

2.4. Immunoblotting and Real Time PCR

Lysates from cultured cells were used for detection of RAGE (GeneTex, Irvine, CA), high-mobility box 1 (HMGB1, Abcam, Cambridge, MA) and β-actin (BD Bioscience) IgG. HRP-conjugated goat anti-rabbit IgG or rabbit anti-mouse IgG secondary antibodies (Santa Cruz Biotechnology) were used to identify sites of binding of the primary antibody. Densitometric analysis was performed using the ImageJ program (version 1.3.8×, NIH, Bethesda, MD). RNA was isolated from HAEC using Trizol (Invitrogen, Carlsbad, CA), followed by reverse transcription using the AffinityScript Reverse Transcriptase (Stratagene, Santa Clara, CA). Real Time PCR was subsequently performed for human RAGE and MCP-1 using Gene Expression Assays (Applied Biosystems, Foster City, CA), normalized to the human GAPDH probe set (Applied Biosystems) and analyzed on an Mx3005P Real Time PCR device (Stratagene). Data were analyzed using the comparative Ct method.

2.5. Data and statistical analysis

All experiments were performed in duplicate wells for each condition and repeated at least three times. Data are presented as mean ± S.D. or as mean fold difference from the non-infected control ± S.D. Representative experiments of 3 independent runs are shown for the immunoblotting studies. Statistical analysis of the murine cell data was performed by means of two-way ANOVA with the type of infection (no infection, *P. gingivalis* 381 or DPG3) and the status of RAGE expression in the endothelial cells (from C57BL/6 or RAGE−/− mice) *or* the time point tested (6 or 24 h after infection) as the two independent variables. Analysis of data from experiments with the human cells was performed by one-way ANOVA with the type of infection or treatment as the independent variable. Pair-wise comparisons between the different groups were subsequently carried out using a *t*-test and the post hoc Ryan–Einot–Gabriel–Welsch multiple range test. p values <0.05 were considered statistically significant.

3. Results

3.1. RAGE levels in primary MAEC upon infection with P. gingivalis

It is well established that RAGE is expressed in endothelial cells and that its activation leads to cellular injury and inflammation [12]. We first explored whether *P. gingivalis* infection further affects levels of RAGE in C57BL/6 MAEC. The effect of infection depended on the time point tested (two-way ANOVA $p = 0.0140$). As seen in Fig. 1a, *P. gingivalis* 381 infection increased RAGE expression in C57BL/6 MAEC by 2.4-fold 6 h after infection compared to the non-infected group ($p = 0.0122$, $n = 3$). At 24 h (Fig. 1b) the magnitude of the increase was smaller, 1.3-fold compared to control ($p = 0.0128$, $n = 3$). As the major fimbriae of *P. gingivalis* are required for the pathogen's adhesion to/invasion of host cells and for virulence, we also tested the effects of its fimbriae-deficient mutant, DPG3. We found a significant increase in RAGE expression in DPG3-infected MAEC compared to non-infected cells at 6 h (2.4-fold, $p = 0.0108$, $n = 3$), but no effect at 24 h ($p = 0.7634$, $n = 3$).

3.2. MCP-1 production by MAEC upon bacterial infection

The effect of infection type on the production of the pro-inflammatory chemokine MCP-1 depended on the status of RAGE expression in the endothelial cells (two-way ANOVA $p = 0.0029$). In