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ORIGINAL ARTICLE

Effects of resveratrol on expression of vascular endothelial growth factor in human gingival fibroblasts stimulated by periodontal pathogens

MARÍA J. NÚÑEZ[1], SILVIA NOVÍO[2], JOSÉ BALBOA[3], JUAN SEOANE[3], JUAN A. SUÁREZ[4] & MANUEL FREIRE-GARABAL[2]

[1]*Department of Nursing, School of Medicine and Nursing and the Departments of* [2]*Pharmacology,* [3]*Oral and Maxillofacial Surgery, and* [4]*Morphological Science, School of Medicine and Dentistry, University of Santiago de Compostela, Santiago de Compostela, Spain*

Abstract

Objective. To investigate the effects of resveratrol, a naturally occurring polyphenol, on the expression of vascular endothelial growth factor (VEGF) in human gingival fibroblast culture in response to vesicles and outer membrane proteins from periodontopathic bacteria. ***Material and methods.*** Human gingival fibroblasts were stimulated with vesicles and outer membrane proteins from *Aggregatibacter actinomycetemcomitans* and *Porphyromonas gingivalis.* In human gingival fibroblast cultures treated with or without resveratrol, VEGF production was evaluated by means of enzyme-linked immunosorbent assay and VEGF mRNA expression by means of reverse transcription polymerase chain reaction analysis. Vascular permeability enhancement was measured by the leakage of intravenously injected dye at the injection site of supernatant from cultures of human gingival fibroblasts stimulated by vesicles and outer membrane proteins. ***Results.*** Resveratrol significantly inhibited the increased production of VEGF by human gingival fibroblasts in response to vesicles and outer membrane proteins from periodontopathic bacteria, as shown by the detection of these proteins and their mRNA *in vitro.* Moreover, resveratrol treatment significantly decreased vascular permeability enhancement induced by supernatant from human gingival fibroblast cultures stimulated by vesicles and outer membrane proteins. ***Conclusions.*** Overall, these findings suggest that resveratrol inhibits production of VEGF by stimulated human gingival fibroblasts and can inhibit vascular permeability, suggesting a therapeutic role for it in pathogenic bacteria-induced periodontal inflammation.

Key Words: *Angiogenesis, periodontal disease, polyphenols, vascular permeability*

Introduction

Vascular endothelial growth factor (VEGF) is a potent inducer of vascular permeability and angiogenesis [1]. Studies by Suthin et al. [2] have shown increased VEGF expression in human gingival fibroblast (HGF) cultures stimulated with lipopolysaccharide (LPS), vesicle (Ve), and outer membrane protein (OMP) from two major etiologic agents of periodontitis: *Aggregatibacter actinomycetemcomitans* and *Porphyromonas gingivalis.* Supernatant of Ve- and OMP-stimulated HGF cultures strongly induced vascular permeability enhancement (VPE). This was markedly suppressed upon simultaneous injection of anti-VEGF polyclonal antibodies with the supernatant. Heating and protease treatment of the stimulants reduced the VEGF-enhancing levels in Ve and OMP *in vitro.* These results suggested that Ve and OMP may be crucial heat-labile and protease-sensitive components of periodontal pathogens that enhance VEGF expression. In addition, VEGF may be associated with the etiology of periodontitis in its early stages due to neovascularization stimulated by periodontal pathogens causing swelling and edema [2].

The ≈5000 different polyphenols that have been described to date have been subdivided into subgroups such as isoflavones, flavonoids and lignans. Recently, several polyphenols have been found to inhibit angiogenesis in several standard angiogenesis models [3]. Bearing in mind that angiogenesis is involved in several diseases, such as diabetic retinopathy [4], cancer, and chronic inflammation [5], the

Correspondence: Manuel Freire-Garabal, Neuroimmunology Laboratory, Department of Pharmacology, School of Medicine, University of Santiago de Compostela, C/ San Francisco, s/n. 15782 Santiago de Compostela, La Coruña, Spain. Tel: +34 981581744. Fax: +34 981573191. E-mail: manuel.freire-garabal@usc.es

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discovery of these polyphenols as angiogenesis inhibitors has shed light on the health-beneficial mechanisms of natural products, which are rich in these molecules. At the molecular level, different studies have provided vital information on how these molecules inhibit endothelial cell growth [6,7]. Perhaps the primary therapeutic advantage of these small natural molecules over large protein compounds is that they can be administrated orally, without causing severe side effects [3].

As the most abundant of these polyphenols, resveratrol has attracted much attention and has been extensively studied both *in vitro* and *in vivo*. Resveratrol is a compound found in grapes and related products, as well as in some nuts. It inhibits both normal and malignant cell proliferation *in vitro* at relatively high concentrations [8]. Recently, resveratrol has been reported to be an angiogenesis inhibitor which is sufficiently potent to suppress fibroblast growth factor (FGF)-2 and VEGF-induced neovascularization *in vivo* [4]. It directly inhibits capillary endothelial cell proliferation, migration, and tube formation *in vitro* [4]. Unlike single angiogenic factor antagonists, the therapeutic value of this molecule is that it blocks a common angiogenic pathway triggered by several angiogenic factors. Another advantage is that this small molecule can be administered orally [3].

The aim of this study was to assess the effects of resveratrol on VEGF expression in HGF culture in response to various components of two major periodontal pathogens, namely *P. gingivalis* and *A. actinomycetemcomitans*.

Material and methods

Cell culture

HGFs were obtained from the healthy gingival tissue of a female patient using the method of Somerman et al. [9]. A gingival biopsy was performed with the patient's informed consent. The resected tissue was immediately immersed and rinsed several times in Dulbecco's modified Eagle's medium formulated with 1 mM sodium pyruvate (DMEM; Gibco BRL, Life Technologies, Barcelona, Spain) containing antibiotic (penicillin: 1000 U/ml; streptomycin: 1250 μg/ml; and amphotericin B: 5 μg/ml). Tissue samples were then cultured in DMEM, and supplemented with 10% fetal bovine serum (FBS; Gibco BRL) containing penicillin (100 U/ml) and streptomycin (125 μg/ml) in a humidified atmosphere of 5% CO_2 and 95% air at 37°C. Outgrowth cells from the tissue cultures were subcultured and used for subsequent assay at passage levels 5–10. The cells did not show alkaline phosphatase activity but did express vimentin, which is one of the fibroblast molecular markers.

Measurement of VEGF protein

HGFs (5×10^3 cells/ml) were seeded into a 96-well cell culture plate (BD, Madrid, Spain) with DMEM supplemented with 10% FBS. Confluent HGF cultures were washed and incubated in FBS-free DMEM for 24 h. Monolayers of HGF were cultured with 50 μg/ml of Ve and OMP from both *A. actinomycetemcomitans* and *P. gingivalis* in 1% FBS–DMEM in the absence (control and placebo) or presence of different concentrations of resveratrol (10, 20, 40, 80, and 160 μM) for periods of 0, 12, 24, and 48 h. Fifty μg/ml concentrations of Ve and OMP were chosen on the basis of the dose-response studies carried out by Suthin et al. [2]. In order to clarify the active components of periodontopathic bacteria, monolayers of HGF were cultured with 50 μg/ml of Ve and OMP from *A. actinomycetemcomitans* and *P. gingivalis* after the stimulants had been heated (100°C for 30 min) or treated with protease K-agarose (Sigma-Aldrich, Madrid, Spain) in 1% FBS–DMEM for 24 h [2]. Thereafter, supernatant was collected from each culture, and the respective VEGF concentrations were determined using a commercial VEGF enzyme-linked immunosorbent assay (ELISA) kit (R&D Systems Vitro, Madrid, Spain) according to the manufacturer's instructions. Briefly, 200 μl of cell supernatant was incubated with 50 μl of buffered protein base for 2 h at room temperature in a 96-well plate coated with a monoclonal antibody against VEGF. After three washings, a conjugate consisting of polyclonal anti-VEGF antibody and horseradish peroxidase was added and incubated for 2 h at room temperature. After the addition of tetramethylbenzidine, absorbance was measured at 450 nm in a Bio-Tek ELx800 microplate reader. For standardization, serial dilutions of recombinant human VEGF were assayed [10].

mRNA expression of VEGF in HGF culture

HGFs (5×10^5 cells/ml) were seeded in 150-mm cell culture dishes (BD) in DMEM supplemented with 10% FBS. Confluent HGF cultures were then washed and incubated in FBS-free DMEM for 24 h. Monolayers of HGF were cultured with 50 μg/ml of Ve and OMP from *A. actinomycetemcomitans* and *P. gingivalis* in 1% FBS–DMEM in the absence (control and placebo) or presence of 80 μM resveratrol (maximum effective dose obtained in previous experiment) for periods of 0, 2, 4, 8, 12, and 24 h. Total RNA was isolated from HGFs using TriZol LS reagent (Gibco BRL), and first-strand cDNA was synthesized with the SuperScript™ Preamplification System (Gibco BRL) according to the manufacturer's instructions. The sets of polymerase chain reaction (PCR) primers used were as follows: VEGF, 5′-GCAGAATCATC ACGAAGTGG-3′ for forward and 5′-GCATGGTGA

TGTTGGACTCC-3′ for reverse; glyceraldehyde-3-phosphate dehydrogenase (GAPDH), 5′-CATCACC ATCTTCCAGGAGC-3′ for forward and 5′-CATGA GTCCTTCCACGATACC-3′ for reverse. The cDNA was amplified for 30 thermal cycles. The PCR working conditions were as follows: denaturation, 95°C for 1 min; annealing, 64°C for VEGF and 60°C for GA PDH for 1 min; and extension, 72°C for 1 min. The PCR products were electrophoresed on agarose gel and stained with ethidium bromide. To control potential variations in gel loading, GAPDH mRNA expression was analyzed. The results, which are expressed as the relative mRNA accumulation, using GAPDH mRNA as an internal standard, were analyzed [11].

VPE assay

Preparation and administration of the culture supernatants. HGFs (5×10^5 cells/ml) were seeded in 150-mm cell culture dishes in DMEM supplemented with 10% FBS. Confluent HGF cultures were washed and incubated in FBS-free DMEM for 24 h. Monolayers of HGF were cultured with 50 μg/ml of Ve and OMP from *A. actinomycetemcomitans* and *P. gingivalis* in 1% FBS–DMEM in the absence (control and placebo) or presence of 80 μM resveratrol for 48 h. Then the culture supernatant was collected and concentrated 100 times by Centriprep-10 and Centricon-10 ultrafiltration devices (10 kDa; Millipore, Bedford, MA) using a bicinchoninic acid protein assay kit (Cultek SL, Madrid, Spain).

Concentrated supernatant (30 μl), in both the presence and absence of 30 μl of anti-VEGF rabbit serum, was inoculated intradermically into the clipped flank of guinea pigs. Skin samples were usually collected 15 min after the dermal exposure.

Measurement of microvascular leakage. The VPE activity of each sample was measured by quantifying the extravasation of Evans blue dye bound to plasma protein, as previously described [12]. Guinea pigs were anesthetized with an intramuscular injection of ketamine (80 mg/kg body weight). Thirty mg/kg body weight of Evans blue (2.5% solution in 0.6% saline) was then administered intravenously, followed by the inoculation of the supernatants as mentioned above. The animals were then killed and the skin samples excised. Activity was expressed in terms of micrograms of released dye. Dye leakage at the injection site of concentrated supernatant from a non-stimulated HGF culture was used as a control.

Determination of oxidative DNA damage

Calf thymus DNA damage was determined using the ethidium-binding assay [13], in which DNA damage is indicated by the failure of the nucleic acid to enhance the fluorescence of ethidium bromide (EB). A total of 3 ml of phosphate-buffered saline (PBS; pH 7.4) containing calf thymus DNA (80 μg/ml) with 80 μM resveratrol (maximum effective dose obtained in previous experiments) and different concentrations of Cu (II) (0, 50, 100, 150, 200, and 250 μM) was incubated at 37°C for 1 h under aerobic conditions. After incubation, 50 μl of 0.75 mg/ml EB was added and the fluorescence was measured by spectrofluorimetry with excitation at 510 nm and emission at 590 nm. As controls, 100% fluorescence was assessed in a solution containing all reagents (including DNA) except for resveratrol. Zero fluorescence was assessed in a solution identical to the 100% reference solution except for DNA. The loss in fluorescence was used as a measure of DNA damage.

Preparation of stimulants

The strains tested came from culture collections of *A. actinomycetemcomitans* Y4 (ATCC 43718) and *P. gingivalis* 2561 (ATCC 33277). Bacterial strains were maintained in brain heart infusion broth containing hemin (5 mg/l) and menadione (0.5 mg/l). The strains were grown at 37°C under anaerobic conditions (N_2:CO_2:H_2; 8:1:1).

Ve was prepared by the method of Grenier & Mayrand [14]. Briefly, bacterial cells were first removed from the growth medium by centrifugation (10 000*g* for 15 min), and then ammonium sulfate was added to the culture supernatant. After a second centrifugation (20 000*g* for 40 min), the pellet was suspended in Tris buffer (50 mM^3, pH 9.5) containing 0.5 mM^3 dithiothreitol. The Ve was then collected by centrifugation (27 000*g* for 40 min) and resuspended in Tris buffer (50 mM^3, pH 7.2) containing 0.5 mM^3 dithiothreitol. A final centrifugation was carried out (27 000*g* for 40 min), and Ve was resuspended in Tris buffer (pH 7.2), lyophilized and kept at –80°C.

OMP was prepared by the method of Boyd & McBride [15]. Freeze-dried bacterial cells were incubated for 30 min at room temperature, sheared by passage through a 26-gauge needle with manual pressure, and mixed in a Waring blender for 10 s. The mixture was then centrifuged at 10 000*g* for 20 min to remove intact cells and debris. OMP was isolated by centrifugation at 80 000*g* for 2 h. The pellet was finally lyophilized.

Drugs

Trans-Resveratrol (trans-3,4,5′-trihydroxystilbene) was purchased from Sigma. For *in vitro* effects, a 100 mM solution of resveratrol was prepared in

dimethyl sulfoxide (DMSO) and all test concentrations were prepared by diluting the appropriate amount of stock solution in tissue culture medium. The concentration of DMSO in culture medium was kept below 0.1% in the resveratrol-treated, placebo and control cell culture media. Resveratrol was added to the cultures at concentrations of 10–160 μM.

Statistical analysis

Statistical analysis was performed using an ANOVA with grouping of means by Student–Newman–Keuls multiple range tests (SPSS 15.0.1; SPSS Inc., Chicago, IL). Differences were considered to be significant when P was < 0.05.

Results

Measurement of VEGF protein

Confluent monolayers of HGF were cultured in 96-well plates and stimulated with 50 μg/ml Ve and OMP from *A. actinomycetemcomitans* and *P. gingivalis* for 48 h. The supernatant from each culture was then collected, and the respective VEGF concentrations were measured by ELISA once in triplicate. As shown in Figure 1, VEGF levels were significantly higher in HGF cultures exposed to Ve and OMP from *P. gingivalis* compared to the unstimulated culture. Enhancement of VEGF production was also observed in HGF cultures exposed to Ve and OMP from *A. actinomycetemcomitans* when compared to the unstimulated culture. The degree of VEGF enhancement attained by Ve and OMP from *P. gingivalis* in the HGF culture (Figure 2) was observed to increase in a time-dependent manner ($P < 0.05$). Identical results were obtained after stimulation with Ve and OMP from *A. actinomycetemcomitans* in the HGF culture (data not shown). Abolition of VEGF induction in the HGF culture was observed following heating and protease digestion of Ve and OMP.

There was a delay in and an inhibition of the VEGF response with resveratrol treatment. Resveratrol significantly inhibited the increased production of VEGF by HGF in response to Ve and OMP from periodontopathic bacteria (Figures 1 and 2).

mRNA expression of VEGF in HGF culture

VEGF mRNA expression following stimulation with 50 μg/ml Ve and OMP from *A. actinomycetemcomitans* and *P. gingivalis* for periods of 0, 2, 4, 8, 12, and 24 h is shown in Figure 3. When the HGFs were probed for VEGF mRNA production by reverse transcription (RT)-PCR, 214-bp bands representative of VEGF were observed from 2 to 24 h after stimulation. The size of these bands was consistent with that of the designed primers. The bands were compared to those for the housekeeping gene GAPDH (286 bp) in order to control variation in gel loading. The peaks of VEGF expression by stimulation with OMP from *A. actinomycetemcomitans* and *P. gingivalis* were observed at 8 h, and the peaks of Ve from *A. actinomycetemcomitans* and *P. gingivalis* were observed at 2 and 4 h, respectively.

Next, we examined whether resveratrol attenuated the increased expression of VEGF by Ve and OMP from periodontopathic bacteria. VEGF mRNA was dramatically reduced by resveratrol treatment. At a concentration of 80 μM, resveratrol inhibited maximum expression of VEGF induced by Ve from *A. actinomycetemcomitans* at 2 h, Ve from *P. gingivalis* at 4 h, and OMP from both bacteria at 8 h to undetectable levels (data not shown).

VPE assay

To ascertain whether supernatant from cultures of Ve- and OMP-stimulated HGF could induce enhancement of vascular permeability *in vivo,* we performed a VPE assay using guinea pigs. As shown in Table I, direct intradermal injection of supernatant from Ve- and OMP-stimulated HGF culture strongly induced VPE ($P < 0.05$). Resveratrol treatment (80 μM) significantly decreased VPE. Furthermore, the observed induction of VPE was markedly suppressed when anti-VEGF polyclonal antibodies were simultaneously injected with the supernatant.

Oxidative DNA damage

It was found that neither resveratrol nor Cu (II) alone showed an appreciable effect on the DNA. Furthermore, the incubation of DNA with Cu (II) ions and resveratrol resulted in the induction of very modest levels of oxidative damage, as indicated by a decrease in the ability of the nucleic acid to enhance the fluorescence of EB (Figure 4).

Discussion

In this study, resveratrol inhibited the increased production of VEGF by HGF in response to Ve and OMP from periodontopathic bacteria, as shown by the detection of these proteins and their mRNA *in vitro.* Moreover, it decreased VPE induced by supernatant from cultures of Ve- and OMP-stimulated HGF. Nevertheless, the analysis of oxidative DNA damage showed that the anti-angiogenetic activity of resveratrol was not related to its pro-oxidant effect.

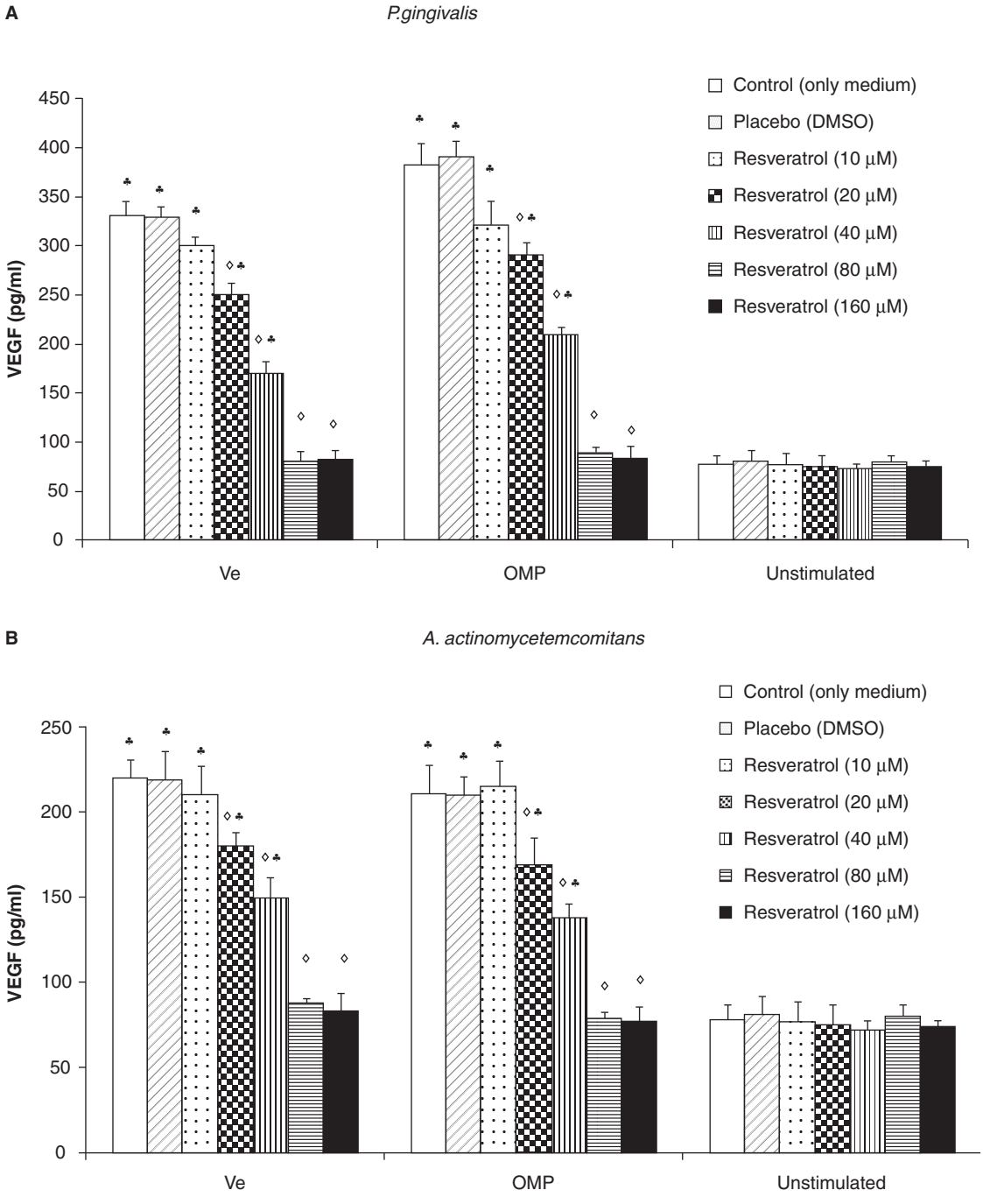

Figure 1. VEGF induction following Ve and OMP stimulation of HGF. Monolayers of HGF were cultured with 50 μg/ml Ve and OMP from (A) *P. gingivalis* and (B) *A. actinomycetemcomitans* in the absence or presence of different concentrations of resveratrol. Supernatant was collected from each culture and respective VEGF concentrations were measured by ELISA once in triplicate. Significant differences with respect to VEGF concentrations were noted between stimulated and unstimulated cells ($^{\clubsuit}P < 0.05$); significant differences in VEGF concentrations were noted between control or DMSO and resveratrol ($^{\diamond}P < 0.05$).
DMEM, Dulbecco's modified eagle medium; DMSO, dimethyl sulfox; FBS, fetal bovine serum; OMP, outer membrane protein; Ve, vesicle.

Angiogenesis is a crucial process in numerous physiological (including embryogenesis, tissue growth, and wound healing) and pathological (such as malignant, ischemic, inflammatory, infectious, and immune diseases) states [16]. Inflammatory cells have been found to take part in the angiogenic process by secreting cytokines that may affect endothelial cell functions. VEGF is an extremely potent and specific angiogenic cytokine which induces proliferation, differentiation, migration and survival of vascular endothelial cells [1]. In addition, VEGF increases vascular permeability leading to extravasation of plasma proteins, fluid accumulation and edema, as well as contributing to an increase in the extension of inflammation [1,17]. It is well known that, during the progression of periodontal disease, the periodontal vasculature is profoundly

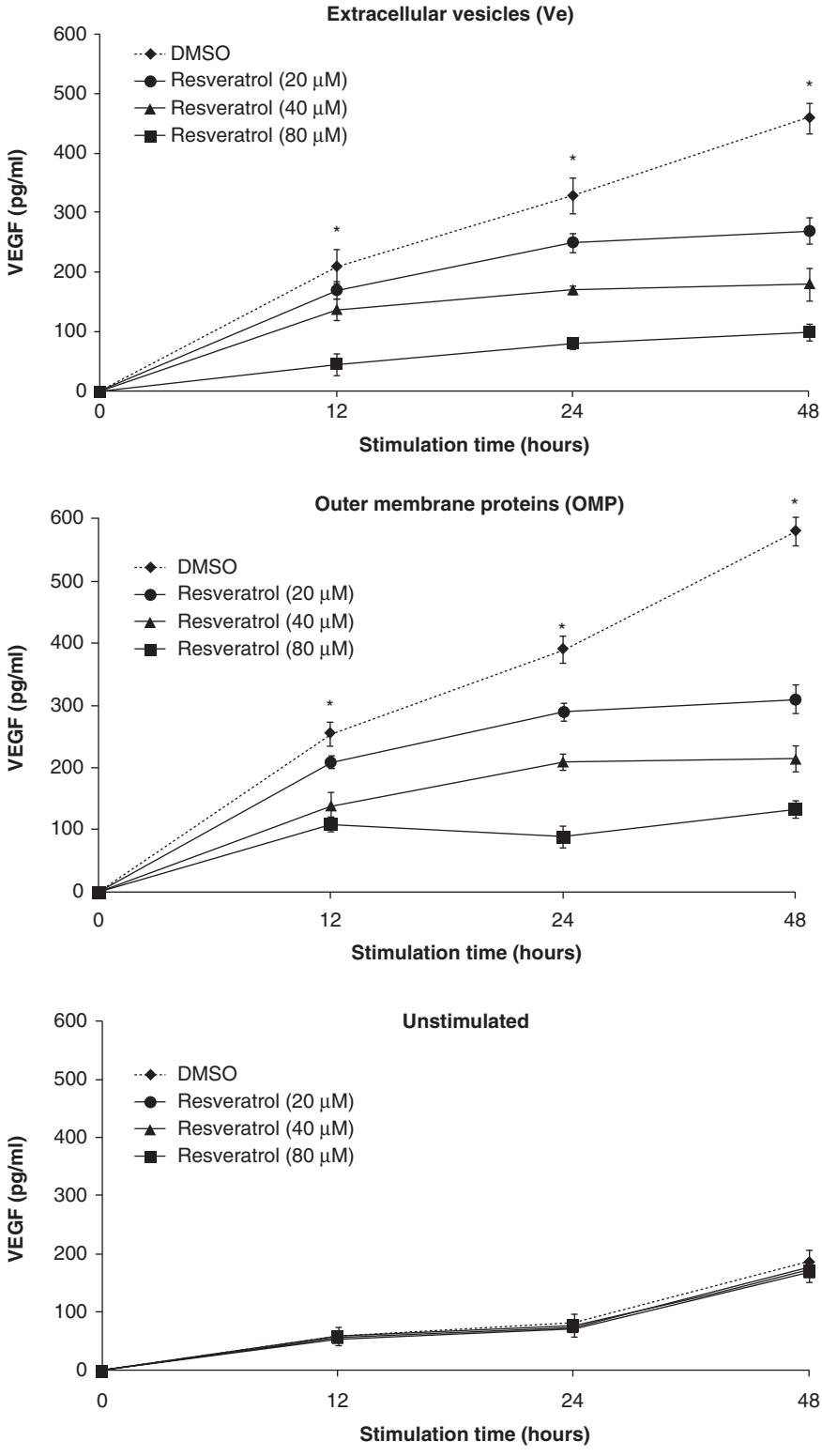

Figure 2. Time course of VEGF induction following Ve and OMP stimulation of HGF. Monolayers of HGF were cultured with 50 μg/ml of Ve and 50 μg/ml of OMP from *P. gingivalis* in the presence of different concentrations of resveratrol for periods of 0, 12, 24, and 48 h. Supernatant was then collected from each culture and respective VEGF concentrations were measured by ELISA once in triplicate. In each case a significant difference was noted between DMSO and resveratrol-treated cells with respect to VEGF induction ($^{\star}P < 0.05$).

affected. Among the angiogenesis markers in periodontal disease, VEGF is one of the most extensively described factors. Several groups of authors have demonstrated that VEGF-induced vascular permeability and angiogenesis are related to periodontal disease [2,10]. VEGF has been detected in human periodontal tissue, gingival crevicular fluid, and saliva and a greater total quantity of VEGF has been found in chronic

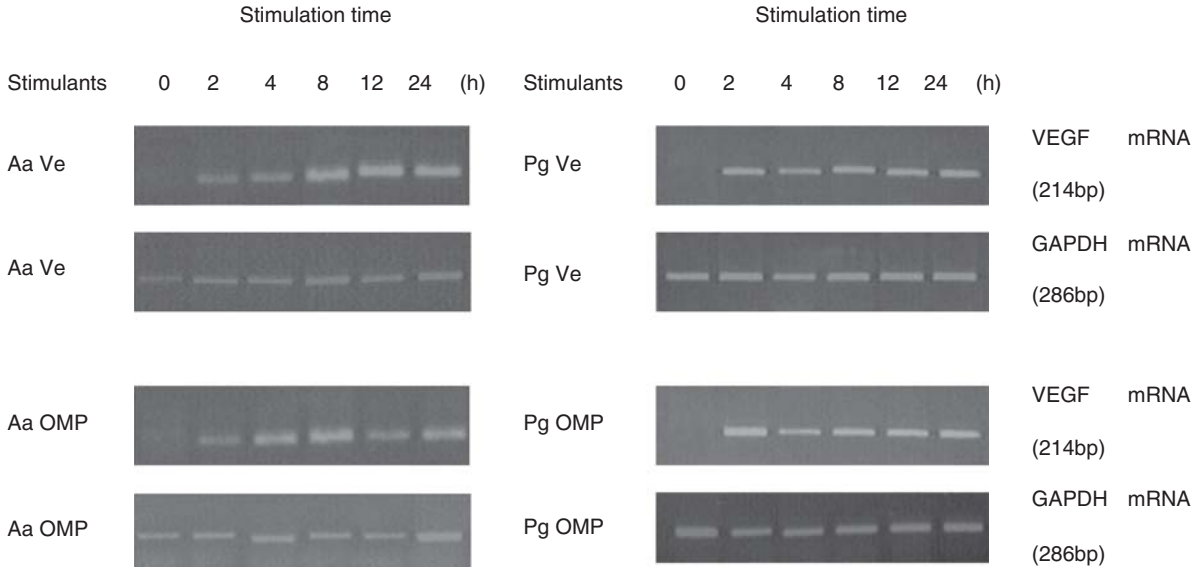

Figure 3. RT-PCR analysis of VEGF mRNA expression in HGF. Confluent HGF cultures were washed and incubated in FBS-free DMEM for 24 h. The cells were washed, fed with DMEM 1% FBS, and incubated with 50 μg/ml Ve and 50 μg/ml OMP from A. actinomycetemcomitans and *P. gingivalis* for periods of 0, 2, 4, 8, 12, and 24 h. Total RNA was extracted and analyzed by RT-PCR analysis. To control variation in gel loading, GAPDH mRNA expression in each lane was also analyzed. Aa = *A. actinomycetemcomitans;* Pg = *P. gingivalis.*

Table I. Vascular permeability-enhancing activity of supernatant obtained from Ve- and OMP-stimulated HGF cultures.

Test specimen	Dye leakage (μg)
VEGF (1 ng/ml)	20.4 (3.2)
VEGF (1 ng/ml)+anti-VEGF antibody	1.9 (0.4)
Anti-VEGF antibody	2.2 (0.1)
Ve (50 μg/ml) from *P. gingivalis*	26.3 (2.0)
Ve (50 μg/ml) from *P. gingivalis*+anti-VEGF antibody	2.1 (0.2)
Ve (50 μg/ml) from *P. gingivalis*+resveratrol (80 μM)	11.8 (3.0)*
Ve (50 μg/ml) from *A. actinomycetemcomitans*	20.3 (1.1)
Ve (50 μg/ml) from *A. actinomycetemcomitans*+anti-VEGF antibody	1.5 (0.3)
Ve (50 μg/ml) from *A. actinomycetemcomitans*+resveratrol (80 μM)	9.6 (1.8)*
OMP (50 μg/ml) from *P. gingivalis*	27.4 (4.6)
OMP (50 μg/ml) from *P. gingivalis*+anti-VEGF antibody	0.9 (0.3)
OMP (50 μg/ml) from *P. gingivalis*+resveratrol (80 μM)	15.9 (2.5)*
OMP (50 μg/ml) from *A. actinomycetemcomitans*	22.5 (3.1)
OMP (50 μg/ml) from *A. actinomycetemcomitans*+anti-VEGF antibody	1.6 (0.4)
OMP (50 μg/ml) from *A. actinomycetemcomitans*+resveratrol (80 μM)	13.1 (1.9)*
Control supernatant	16.2 (3.0)
Control supernatant+anti-VEGF antibody	0.7 (0.1)
Control supernatant+resveratrol (80 μM)	14.0 (2.7)

Monolayers of HGF were cultured with 50 μg/ml Ve and OMP from *A. actinomycetemcomitans* and *P. gingivalis* in 1% FBS–DMEM in the absence or presence of 80 μM resveratrol for 48 h. Supernatant was collected from each culture and concentrated 100 times. VPE activity within the culture supernatant was determined by quantitatively measuring the amount of extravasated Evans blue dye, as described in the Material and methods. In each case a significant difference was noted between groups treated with and without resveratrol with respect to VEGF induction (*$P < 0.05$). The value denotes the mean ± SD ($n = 3$).

periodontitis sites as compared to periodontally healthy sites [18]. Furthermore, Prapulla et al. [19] found decreased VEGF levels in gingival crevicular fluid in patients with periodontitis after periodontal treatment. Therefore, VEGF can be considered as a key biomarker in the progression of periodontal disease [17].

P. gingivalis and *A. actinomycetemcomitans* are major etiological agents in adult and juvenile periodontal

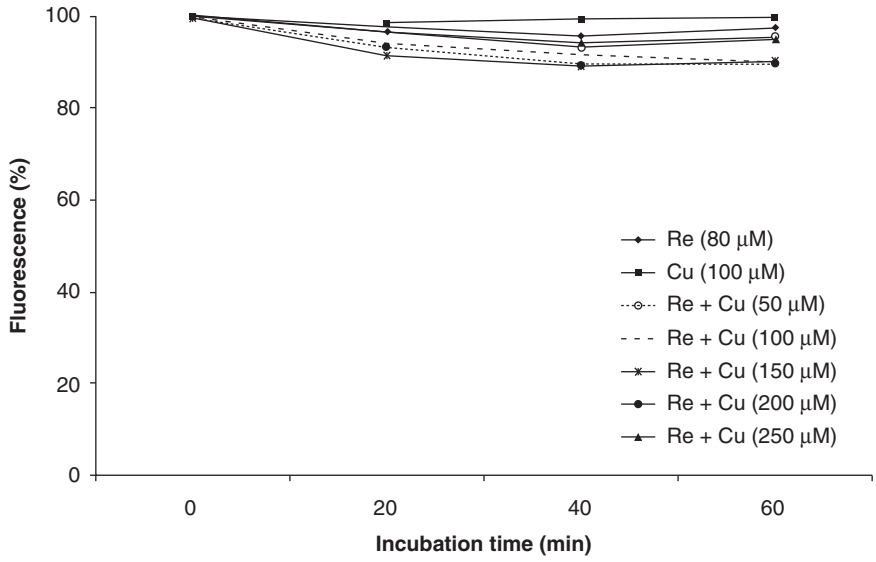

Figure 4. Extent of calf thymus DNA damage induced by resveratrol in the presence of Cu (II) ions. DNA (80 μg/ml) was incubated at 37°C for 60 min with 80 μM of resveratrol and different concentrations of Cu (II) (0, 50, 100, 150, 200, and 250 μM). The integrity of the DNA was then determined by its ability to enhance the fluorescence of EB, as described in the Material and methods. Values are means from three experiments. Re = resveratrol.

disease, and Ve and OMP are important virulence factors. In this study, we found that Ve and OMP derived from these periodontal pathogens had similar effects on the production of VEGF by HGF. Nevertheless, according to Suthin et al. [2], Ve and OMP from *P. gingivalis* influence the production of VEGF in HGF to a greater extent than Ve and OMP from *A. actinomycetemcomitans.* Likewise, semiquantitative analyses confirmed that the mRNA level for VEGF was significantly higher in *P. gingivalis*-lipopolysaccharide-stimulated neutrophils than in *A. actinomycetemcomitans* [20]. Although *a priori,* these results could suggest that the role played by *P. gingivalis* in the pathogenesis of periodontal disease might be more crucial than that of *A. actinomycetemcomitans,* the fact that women are more resistant to some periodontal pathogens and have significantly less clinical attachment loss than men [21], could explain this difference.

Resveratrol has an anti-angiogenetic effect, although the underlying mechanisms have yet to be ascertained. Resveratrol has been found to suppress VEGF expression in cancer cells [22] and to inhibit the binding of VEGF to human umbilical vein endothelial cells [23], the capillary-like tube formation by endothelial cells [23], and vascular endothelial cell growth and proliferation [4,22,23]. The inhibition of endothelial cell proliferation may entail the suppression of vessel formation, at least in an indirect and partial manner. On the other hand, previous *in vivo* studies have indicated that resveratrol inhibits corneal neovascularization induced by VEGF and basic FGF in a dose-dependent manner [4], and suppresses angiogenesis [22].

The mitogen-activated protein kinase (MAPK) signaling pathway is at least one of the targets of resveratrol in endothelial cells, since it effectively inhibits phosphorylation of MAPKs such as p38 MAPK [4,24]. Resveratrol upregulates p21WAF in endothelial cells, which downregulates cyclin D1, -D2 and -E as well as cyclin-dependent kinase (cdk)-2, -4 and -6, leading to cell cycle arrest at the G1 phase [25]. Its inhibitory activity can also be extended to the suppression of proteases such as matrix metalloproteinases, which are proangiogenic factors [26]. It should be noted that even the anti-oxidative effect of resveratrol has been linked to anti-angiogenesis. Reduction of oxidative stress by resveratrol leads to suppression of reactive oxygen intermediate generation, and to the inhibition of activation of transcription factors such as activator protein-1 and nuclear factor-κB [27], which regulate the expression levels of one of the key angiogenic factors, i.e. VEGF [28]. Thus, the anti-angiogenic mechanisms of resveratrol are complex. However, the anti-angiogenic role of resveratrol is not only restricted to pathological angiogenesis, but also influences physiological angiogenesis, e.g. the dosages used for the suppression of tumor growth in mice effectively delayed wound repair in normal mice [4]. Therefore, resveratrol cannot discriminate physiological angiogenesis from pathological blood vessel growth. Unlike single angiogenic factor antagonists, the therapeutic value of this molecule is that it blocks a common angiogenic pathway, which is triggered by several angiogenic factors. Another advantage is that this small molecule can be administered orally.

In summary, this study has shown that Ve and OMP are bioactive components of *P. gingivalis* and *A. actinomycetemcomitans* which enhance production of VEGF by HGF. In addition, resveratrol was

demonstrated to inhibit production of VEGF by stimulated HGFs, being able to reduce the vascular permeability. The clinical implications of these findings are promising given that resveratrol can be considered as a therapeutic agent which can play a vital role in the protection of periodontal tissues from the deleterious effects of periodontal pathogens.

Declaration of interest: The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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