Induction of vascular endothelial growth factor expression in human pulp fibroblasts stimulated with black-pigmented *Bacteroides*

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Abstract

Yang L-C, Tsai C-H, Huang F-M, Su Y-F, Lai C-C, Liu C-M, Chang Y-C. Induction of vascular endothelial growth factor expression in human pulp fibroblasts stimulated with black-pigmented *Bacteroides*. *International Endodontic Journal*, **37**, 588–592, 2004.

Aim To investigate the effect of black-pigmented *Bacteroides* on the expression of vascular endothelial growth factor (VEGF) gene in human pulp fibroblasts.

Methodology The supernatants of *Porphyromonas endodontalis*, *Porphyromonas gingivalis* and *Prevotella intermedia* were used to evaluate VEGF gene expression in human pulp fibroblasts. The levels of mRNAs were measured by the quantitative reverse-transcriptase polymerase chain reaction analysis.

Results Black-pigmented *Bacteroides* induced significantly high levels of VEGF mRNA gene expression in human pulp fibroblasts ($P < 0.05$). In addition, the expression of VEGF depended on the bacteria tested.

Conclusions Black-pigmented *Bacteroides* may be involved in developing pulpal disease through the stimulation of VEGF production that would lead to the expansion of the vascular network coincident to progression of the inflammation.

Keywords: black-pigmented *Bacteroides*, pulp fibroblasts, vascular endothelial growth factor.

Received 21 October 2003; accepted 22 April 2004

Introduction

The development and spread of pulpal inflammation is related to a number of factors. Injury to the pulp occurs as a consequence of bacterial infection due to caries, trauma, or an iatrogenic cause (Stashenko *et al.* 1998). Black-pigmented *Bacteroides* such as *Prevotella* and *Porphyromonas* have been found in pulpal lesions (Van Winkelhoff *et al.* 1985, Jim *et al.* 1989, Sundqvist *et al.* 1989). These bacteria play a significant role in the development of clinical symptoms through the production of inflammatory mediators (Chang *et al.* 2003a, Yang *et al.* 2003a,b) and degradative enzymes (Chang *et al.* 2002,Yang *et al.* 2003c). Despite these investigations, the molecular mechanisms involved in the spread of the inflammatory reaction have not been clarified.

An increase in vascular permeability is involved in the active phase of pulpitis as well as in inflammation elsewhere in the body. In the case of pulpitis, an excessive increase of vascular permeability may result in oedema and necrosis, due to the specific anatomic characteristics of the pulp tissue. Various inflammatory substances such as histamine, bradykinin, serotonin, prostaglandin, and leukotriene are known as mediators that increase vascular permeability in pulp tissue (Kim *et al.* 1992). Vascular endothelial growth factor (VEGF) has been identified as an endothelium-specific mitogen and the inducer of angiogenesis in the *in vivo* test systems (Starri *et al.* 1995,Breier 2000). VEGF has the ability to increase vascular permeability, which contributes to increase and extend of inflammation (Johnson *et al.* 1998). VEGF has been found to be increased in inflamed pulp tissue (Artese *et al.* 2002)

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and periapical lesions (Leonardi *et al.* 2003). The possibility that VEGF is produced by pulp fibroblasts may have important implications for the study of pulpal disease and its progression, as well as the healing progress that follows endodontic treatment. However, there have been relatively few studies addressing the presence and expression of VEGF at the pulpal tissue of following bacterial infection.

Pulp fibroblasts are considered as cells primarily concerned with providing physical barriers and structural components in pulpal tissues. The purpose of this *in vitro* study was to investigate the effect of the supernatants of black-pigmented *Bacteroides* (*Porphyromonas endodontalis*, *Porphyromonas gingivalis* and *Prevotella intermedia*) on VEGF mRNA genes expression in human pulp fibroblasts by reverse-transcriptase polymerase chain reaction (RT-PCR) assay.

Materials and methods

Cell culture

Human pulp fibroblasts were cultured using an explant technique as described previously (Chang *et al.* 2000,Chang & Chou 2001). Briefly, impacted third molars were obtained from healthy patients of the Oral Medicine Centre, Chung Shan Medical University Hospital, Taichung, Taiwan. Teeth were sectioned horizontally below the cementoenamel junction with a number 330 high-speed bur with water spray. The pulp tissue was removed aseptically in a laminar flow, and rinsed with Hanks' buffered saline solution, and placed in a 60 mm dish. Pulp tissue was minced with a blade into small fragments and grown in Dulbecco-modified Eagle's medium (DMEM) (Gibco Laboratories, Grand Island, NY, USA) supplemented with 10% foetal calf serum (FCS) (Gibco Laboratories) and antibiotics (100 U ml^{-1} penicillin, 100 μg ml^{-1} streptomycin and 0.25 μg ml^{-1} of fungizone). Cultures were maintained at 37 °C in a humidified atmosphere of 5% CO_2 and 95% air. Confluent cells were detached with 0.25% trypsin and 0.05% EDTA for 5 min, and aliquots of separated cells were subcultured. Cell cultures between the third and eighth passages were used in this study.

Bacterial strains and preparation of supernatants

The bacterial strains tested were *P. endodontalis* (ATCC 27067), *P. gingivalis* (ATCC 33277) and *P. intermedia* (ATCC 25611). They were maintained in brain-heart infusion broth prereduced anaerobically, sterilized, and supplemented with 5 mg l^{-1} haemin and 0.5 mg l^{-1} menadione. The density of the inoculum, prepared in brain–heart infusion broth, was adjusted to a turbidity of 2 McFarland standard (6×10^8 colony-forming units ml^{-1}). After centrifugation, supernatants were filter-sterilized using a 0.2 μm filter and stored at −80 °C until used. The supernatants of *P. endodontalis*, *P. gingivalis* and *P. intermedia* were directly diluted in culture medium and the final dilution was 1 : 100.

Treatments

Confluent cells were trypsinized, counted, and plated at a concentration of 1×10^5 cells in a 60 mm dish and allowed to achieve confluence. Cells arrested in G_0 by serum deprivation (0.5% FCS for 48 h) were generally used in these experiments (Chang *et al.* 2003a,b). Before treatment, the cells were washed with serum-free DMEM and immediately exposed for the indicated incubation times (2, 6 and 24 h) to the supernatants of *P. endodontalis*, *P. gingivalis* and *P. intermedia*, respectively. In addition, cultures with 0.5 and 10% FCS were used as negative and positive control, respectively.

Total RNA preparation and RT-PCR

Total RNA was prepared using TRIzol reagent (Gibco Laboratories) following the manufacturer's instructions. Single-stranded DNA was synthesized from RNA in a 15 μl reaction mixture containing 100 mg random hexamer and 200 units of Moloney murine leukaemia virus reverse transcriptase (Gibco Laboratories). The reaction mixture was diluted with 20 μl of water and 3 μl of the diluted reaction mixture was used for the PCR. PCR reaction mixture contains 10 pmol of forward and reverse primers and 2 units of Tag DNA polymerase. Amplification was performed at 25 cycles for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and 30 cycles for VEGF in a thermal cycle. Each cycle consisted of 1 min of denaturation at 94 °C, 1 min of annealing at 57 °C, and 1 min of extension at 72 °C. The sequences of primers employed are listed in Table 1 (Suthin *et al.* 2003). The PCR products were analyzed by agarose gel electrophoresis.

When the cells were probed for VEGF mRNA production by RT-PCR, a 214 bp band for VEGF was noted. These bands were consistent with the size as designed by the primer. When the band densities were

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measured and compared with the density of the band obtained for the housekeeping gene GAPDH, relative proportions of mRNA synthesis could be determined within each experiment. The intensity of each band after normalization with GAPDH mRNA was quantified by the photographed gels with a densitometer (Alpha-Imager 2000; Alpha Innotech, San Leandro, CA, USA). Each densitometric value was expressed as the mean ± SD.

Statistical analysis

All assays were repeated three times to ensure reproducibility. Statistical analysis was carried out by one-way analysis of variance (ANOVA). Tests of differences of the treatments were analyzed by Duncan's test and a value of $P < 0.05$ was considered statistically significant.

Results

Black-pigmented *Bacteroides* were found to induce VEGF gene expression in human pulp fibroblasts (Fig. 1). However, human pulp fibroblasts resting in 0.5% FCS did not express any detectable level of VEGF gene. Densitometric analysis of the VEGF mRNA gene expression, after normalization by GAPDH, demonstrated that VEGF mRNAs increased about 1.4-, 3.9- and 1.0-fold after exposure to *P. endodontalis* for 2, 6 and 24 h, respectively (Fig. 2). The levels of the VEGF mRNAs increased about 1.5-, 1.0- and 3.7-fold after exposure to *P. gingivalis* for 2, 6 and 24 h, respectively (Fig. 2). The levels of the VEGF mRNAs increased about 1.3-, 1.8- and 1.2-fold after exposure to *P. intermedia* for 2, 6 and 24 h, respectively (Fig. 2).

Discussion

Angiogenesis is defined as the process by which new blood vessels are produced by sprouting from established vessels. Inflamed pulpal tissues have evidence of enhanced expression of inflammatory mediators (Barkhordar *et al.* 1999,Huang *et al.* 1999), many of which can promote angiogenesis, however, VEGF is reported to be the most potent and specific. VEGF plays an important role in the progression of inflammation (Dvorak *et al.* 1995,Ito *et al.* 1995). Therefore, its importance in the complex cascade of events associated with inflammation mediated pulpal tissue destruction cannot be discounted. In this study, VEGF augmented the production of VEGF in response to the supernatants of black-pigmented *Bacteroides* (*P. endodontalis*, *P. gingivalis* and *P. intermedia*) in human pulp fibroblasts by RT-PCR assay. This appears to be the first study to report such an effect of black-pigmented *Bacteroides* on VEGF mRNA gene expression in human pulp fibroblasts. These findings suggest that black-pigmented *Bacteroides* may be involved in developing pulpal disease through the induction of VEGF production. Furthermore, the regulation of VEGF expression occurs at transcriptional levels.

The results of the present study are in agreement with Suthin *et al.* (2003), who demonstrated that VEGF was augmented by the vesicle and out membrane protein from *P. gingivalis* in human gingival fibroblast, but not by lipopolysaccharides (LPS) stimulation using enzyme-linked immunosorbent assay (ELISA). Matsushita *et al.* (1999) have shown that VEGF mRNA and protein were markedly enhanced upon stimulation with LPS from *P. intermedia* in human pulp cells. Botero *et al.* (2003) found that the stimulation with LPS from

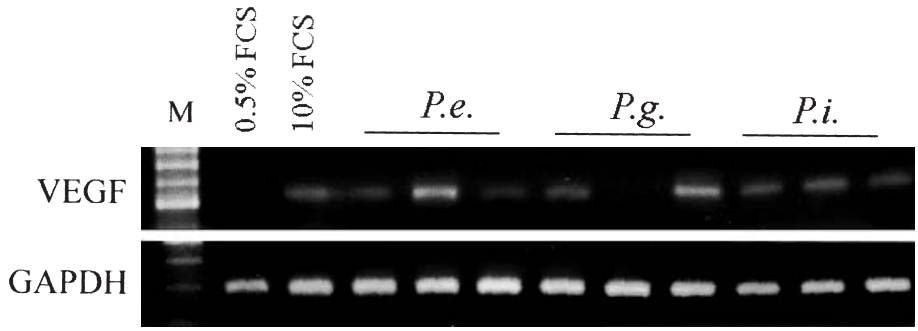

Figure 1 Expression of VEGF mRNA gene stimulated with black-pigmented Bacteroides (*P.e.*, *P.g.* and *P.i.*) in human pulp fibroblasts by RT-PCR assays. M = DNA molecular size marker. Lanes 1 and 2 represent controls: 0.5% FCS and 10% FCS. Lanes 3 to 5 represent *P.e.*: 2, 6 and 24 h, respectively. Lanes 6 to 8 represent *P.g.*: 2, 6 and 24 h, respectively. Lanes 9 to 11 represent *P.i.*: 2, 6 and 24 h, respectively.

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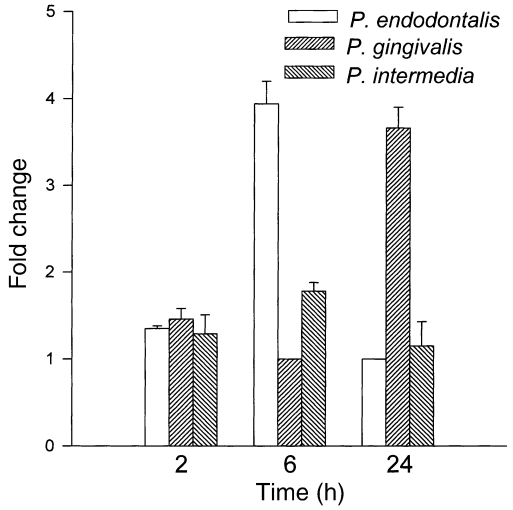

Figure 2 Levels of VEGF mRNA gene treated with black-pigmented *Bacteroides* were measured by AlphaImager 2000. Optical density values expressed as the mean ISD, was obtained from three independent experiments.

P. intermedia can induce VEGF in mouse odontoblast MDPC-23 cells as well as marcrophage RAW 267.4, but not in mouse undifferentiated pulp cell line OD-21 cells and mouse gingival fibroblasts by ELISA. Taken together, the expression of VEGF depended on the bacteria tested and the cell culture system used.

The different activation patterns responsible for the VEGF expression by black-pigmented *Bacteroides* may be explained as follows. First, this activation may result from the different origins of the cells, and the molecular specificity to cellular receptors of stimulation may not necessarily be comparable in all tissues. Second, many of the cell components that have been shown to function as virulence factors in gram-negative bacteria are associated with the bacterial surface. LPS has been characterized as one such molecule that mediates a number of biological activities that can lead to the destruction of host tissue. Moreover, LPS is responsible for many, if not all, of the biological activities exhibited by bacterial byproducts. Last, differences in the chemical structure of lipid A production of the LPS molecule has been demonstrated to affect the intensity of the responses in different systems (Darveau 1998).

The production of VEGF by human pulp fibroblasts exposed to the supernatants of black-pigmented *Bacteroides* probably indicates a role for this protein. Pulp fibroblasts may be expected to produce significant amounts of VEGF in response to endodontic bacteria contributing to the inflammatory response of pulpal lesions. VEGF induces not only increased vascular permeability, but also the chemotaxis of monocytes/macrophages (Clauss *et al.* 1996). An upregulated production of VEGF in pulpal tissue may therefore enhance vascular permeability and the accumulations of inflammatory cells and increase the blood pressure, which results in the irreversible inflammation of pulpal tissues.

To probe this concept further, additional studies regarding the role of VEGF in the pathogenesis of pulpal diseases are needed to determine the relationship between VEGF and pulpal disease. The results of this study suggest that VEGF may be associated with the aetiology of pulpal disease in its early stages, in so far as it is related to neovascularisation stimulated by endodontic pathogens, thereby causing swelling and oedema.

Conclusions

The present study demonstrated that VEGF gene expression by human pulp fibroblasts was induced by black-pigmented *Bacteroides* (*P. endodontalis*, *P. gingivalis* and *P. intermedia*). As up-regulation of VEGF production has been connected with several inflammatory diseases, the results suggest that black-pigmented *Bacteroides* may be involved in developing pulpal inflammation through the stimulation of VEGF production *via* promoting expansion of the vascular network coincident to progression of the inflammation.

Acknowledgements

This study was supported by research grants from the Chung Shan Medical University (CSMU 92-OM-B-047).

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