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Confocal immunolocalization of VE-cadherin- and CXC chemokine-expressing endothelial cells in periapical granulomas

O. Takeichi[1,2], S. Hama[1], K. Iwata[3,4] & K. Ito[2,5]

[1]Department of Endodontics, Nihon University School of Dentistry, Chiyoda-ku, Tokyo; [2]Division of Advanced Dental Treatment, Dental Research Centre, Nihon University School of Dentistry, Chiyoda-ku, Tokyo; [3]Department of Physiology, Nihon University School of Dentistry, Chiyoda-ku, Tokyo; [4]Division of Functional Morphology, Dental Research Centre, Nihon University School of Dentistry, Chiyoda-ku, Tokyo; and [5]Department of Periodontology, Nihon University School of Dentistry, Chiyoda-ku, Tokyo, Japan

Abstract

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Aim To determine whether endothelial cells (ECs) in periapical granulomas can express vascular endothelial (VE)-cadherin, CXCL8 and CXCL10 by examining with two-colour confocal laser scanning microscope.

Methodology Periapical lesions were surgically removed from patients with chronic periapical periodontitis (n = 20), and the paraffin-embedded sections were prepared after being fixed with cold acetone. The 7-μm-thick sections were stained with haematoxylin–eosin and then examined pathologically using a light microscope. The lesions diagnosed as periapical granulomas (17 specimens) were analysed further using immunofluorescence and antibodies specific for human VE-cadherin, CXCL8, and CXCL10. The slides were carefully examined using a confocal laser scanning microscope. The numbers of positive ECs were counted, and the comparison between VE-cadherin-positive ECs and CXCL8 or CXCL10 was assessed statistically using one-way ANOVA followed by a Student–Newman–Keuls test.

Results The expression of CXCL8 and CXCL10 by ECs was detected in 60.4 ± 13.4 and 67.2 ± 13.9%, respectively. However, the percentage of VE-cadherin-expressing ECs was 40.4 ± 10.5%, which was significantly lower ($P < 0.01$) than CXCL8 and CXCL10-expressing ECs. Two-colour immunofluorescence staining revealed that ECs co-expressed VE-cadherin and CXCL8 (37.4 ± 14.1%) or CXCL10 (39.1 ± 13.8%).

Conclusions VE-cadherin expression in ECs was lower than CXCL8 and CXCL10, suggesting that inflamed ECs in periapical granulomas could increase vascular permeability and that leukocyte chemotaxis mediated by ECs might occur. These findings may suggest the possibility that ECs could play a pivotal role in cell recruitment in periapical granulomas.

Keywords: confocal laser scanning microscopy, CXCL10, CXCL8, endothelial cells, periapical granulomas, VE-cadherin.

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Introduction

Periapical granulomas are chronic inflammatory lesions caused by complex polymicrobial infections. They are typified by damage to supporting tissue, including alveolar bone resorption around the apical areas, and by granulomatous tissue with a large number of inflammatory cells, such as macrophages, lymphocytes, plasma cells and polymorphonuclear leukocytes (PMNs) (Simon 1994). Migration of inflammatory cells, which predominantly express various cytokines (Tani-Ishii *et al.* 1995, Takeichi *et al.* 1998, Ataoglu *et al.* 2002) and growth factors (Marton & Kiss 2000) that augment immune responses, seem to play

Correspondence: Osamu Takeichi, DDS PhD, Department of Endodontics, Nihon University School of Dentistry, 1-8-13, Kanda-Surugadai, Chiyoda-ku, Tokyo 101-8310, Japan (Tel.: 81 3 3219 8142; fax: 81 3 3219 8348; e-mail: takeichi@dent.nihon-u.ac.jp).

an important role in the progression of periapical lesions. In particular, infiltrating activated macrophages and lymphocytes are believed to initiate periapical inflammation, eventually leading to progressive bone loss (Nair 2004).

The chemokine family consists of small peptides known to mediate the recruitment of leukocytes; these peptides are classified into four major groups: CXC (or α), CC (or β), CX_3C, and C (Rossi & Zlotnik 2000). The CXC and CC subfamilies are subdivided further according to structural homologies and biological activities. For instance, interleukin 8 (IL-8) and interferon γ-inducible protein 10 kDa (IP-10) are CXC chemokines that are expressed by endothelial cells (ECs) and named CXCL8 and CXCL10, respectively. CXCL8 is upregulated in periapical lesions (Marton *et al.* 2000) and can be expressed by mononuclear cells isolated from such lesions (Lukic *et al.* 2006). In addition, expression levels of CXCL10, CCL5 (RANTES; regulated on activation, normal T-cell expressed and secreted protein) and CCL2 (MCP-1; monocyte chemoattractant protein-1) are significantly higher in apical cysts than in granulomas (Silva *et al.* 2005). Thus, chemokines may be important in promoting the entry of leukocytes into periapical lesions during immune-mediated inflammation.

Interestingly, chemokines selectively attract leukocyte subsets and activate binding of leukocyte integrins to cell adhesion molecule counter receptors on ECs (Farber 1997, Rossi & Zlotnik 2000, Luther & Cyster 2001). CXCL8 is a key chemoattractant factor for and recruiter of PMNs (Oido-Mori *et al.* 2001). It also activates PMN functions, such as release of lysosomal enzymes and generation of superoxide anions (Cross *et al.* 2003). CXCL10 is expressed by various inflammatory cells as well as ECs and attracts T-helper type 1 cells (Loetscher *et al.* 1996). Thus, CXCL8 and CXCL10 may function in the initiation and progression of periapical diseases.

As blood flows through blood vessels, the vascular ECs lining the vessels are constantly exposed to circulating leukocytes. Endothelial permeability to circulating cells is controlled in part by intercellular junctions, such as adherens junctions. The transmembrane proteins responsible for cell–cell adhesion in adherens junctions belong to the cadherin superfamily (Corada *et al.* 1999, Rahimi & Kazlauskas 1999). The vascular endothelial (VE)-cadherin could be potent regulators of vascular permeability at adherens junctions (Carmeliet *et al.* 1999); migration of leukocytes across endothelial adherent junctions may be regulated by the VE-cadherin and CXC chemokines that are expressed by ECs.

The mechanisms of inflammatory reactions in periapical lesions are partly understood, however, the role of local immune systems in the pathogenesis of periapical granulomas still remains controversial. Although mononuclear cells have been a major focus of cell migration studies, ECs might be more important in persistent leukocyte migration, as they play a central role in cell–cell communication with leukocytes in blood flow. A complete analysis of their functions is necessary to determine the mechanisms of host defences against virulence factors, such as bacterial lipopolysaccharide, proteinase or other pathogens.

In this study, the ability of ECs in periapical granulomas to express both VE-cadherin and CXCL8 or CXCL10 was examined. Two-colour immunohistochemical analysis using VE-cadherin polyclonal and CXCL8 or CXCL10 monoclonal antibodies was performed, and confocal laser scanning microscopy was used to localize the immunopositive cells.

Materials and methods

Human subjects

The samples used in this study were taken from patients diagnosed with chronic apical periodontitis without acute features, such as throbbing pain, swelling of mucosa around tooth apices, or other symptoms. In total, 20 patients (12 male, 8 female) ranging in age from 30 to 65 years were examined. No systemic disease was observed in any of the patients, and they had not taken antibiotics within the previous 6 months. The study protocol was approved by the ethics committee of the Nihon University School of Dentistry, as required by the Declaration of Helsinki. The nature of the procedure and the possible discomforts and risks were fully explained to the patients prior to the collection of samples, and written consent was obtained from all participating patients.

Tissue preparation

Periapical lesions were obtained from the patients at the time of a surgical treatment (root-end resection and root-end filling) and immediately fixed with neutral buffered formalin. After the fixed tissues were embedded in paraffin, 7-μm-thick sections were prepared on poly-L-lysine-coated slides. Sectioned areas of embedded samples were randomly selected for analysis, and at

least three different places were sectioned for each sample block.

Histological examination of periapical lesions

Paraffin sections of the surgically obtained periapical lesions were deparaffinized with xylene and rehydrated with graded ethanol. Rehydrated sections were then stained with haematoxylin–eosin, and their histology was examined under a light microscope (BH-2; Olympus, Tokyo, Japan).

Two-colour immunofluorescent staining and confocal laser scanning

To localize VE-cadherin- and chemokine-expressing cells in periapical granulomas, two-colour immunofluorescent staining of the paraffin sections was performed using two primary antibodies. In brief, the paraffin sections were incubated with 10% normal rabbit serum (Vector Laboratories Inc., Burlingame, CA, USA) for 20 min to block nonspecific binding. The sections were then incubated for 4 h with a goat polyclonal antihuman VE-cadherin antibody [1 : 200 in phosphate buffered saline (PBS); Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA] and a mouse monoclonal antihuman chemokine antibody. The chemokine antibody was specific for either human CXCL8 (1 : 20; R&D Systems Inc., Minneapolis, MN, USA) or human CXCL10 (1 : 10; Abcam PLC, Cambridge, UK). After incubation, the sections were washed three times with PBS and then incubated with a tetramethyl rhodamine isothiocyanate (TRITC)-conjugated rabbit antigoat IgG antibody (1 : 300; Cappel, Solon, OH, USA) and an fluorescein isothiocyanate (FITC)-conjugated donkey antimouse IgG antibody (1 : 300; Jackson ImmunoResearch, West Grove, PA, USA) to detect VE-cadherin and CXCL8 or CXCL10, respectively. Nuclear counterstain was also performed using TO-PRO-3 (Invitrogen, Carlsbad, CA, USA). After a final wash with PBS, the sections were mounted with aqueous mounting medium (PermaFluor, Marseille, France), and cover-slipped.

Immunopositive ECs were carefully examined using a confocal laser scanning microscope (LSM 510; Carl Zeiss Co. Ltd., Oberkochen, Germany) equipped with argon and helium–neon lasers producing monochromatic light at 488 and 543 nm, respectively. Each FITC–TRITC preparation was scanned twice with filter changes between the scans; one scan served to excite FITC (488 nm), and the other scan served to excite TRITC (543 nm). As a negative control, normal rabbit IgG antibodies (Cayman Chemical, Ann Arbor, MI, USA) were used in place of the primary antibodies described above.

Statistical analysis

For each antibody, the numbers of immunostained ECs were counted over five different fields of vision in one section by two examiners at a magnification of 200×. The results are expressed as the mean percentage of immunopositive ECs relative to the total number of ECs. Statistical analysis was performed using SPSS version 15.0 for Windows (SPSS Inc., Chicago, IL, USA). Differences amongst VE-cadherin- and CXCL8- or CXCL10-positive ECs were analysed using one-way ANOVA followed by a Student–Newman–Keuls test. Differences were considered significant at $P < 0.01$ or $P < 0.05$.

Results

Histological examination of periapical lesions

Paraffin sections of surgically obtained periapical lesions ($n = 20$) were examined histologically using haematoxylin–eosin staining to define the pathological features. The criteria stated by Nair (1998) were used to evaluate the lesions. Cellular characterization was assessed by morphological appearance. Of the 20 periapical lesions examined by light microscopy, 17 showed granulomatous tissues with a large number of infiltrating cells (macrophages, plasma cells, lymphocytes, fibroblasts and PMNs). No epithelial cells were observed in any views of these samples. These specimens were diagnosed as periapical granulomas (Fig. 1a) and selected for analysis by confocal laser scanning microscopy. The remaining three periapical lesions represented radicular cysts characterized by an epithelium-lined cavity (Fig. 1b), and were excluded from the study.

Immunohistochemical analysis for VE-cadherin, CXCL8 and CXCL10

To examine the involvement of VE-cadherin- and CXCL8- or CXCL10-expressing cells in periapical granulomas, paraffin sections of periapical lesions ($n = 17$) were analysed using a polyclonal antibody specific for human VE-cadherin or a monoclonal antibody specific for human CXCL8 or CXCL10. ECs were identified by nuclear counter-stain using TO-PRO-3 (data not

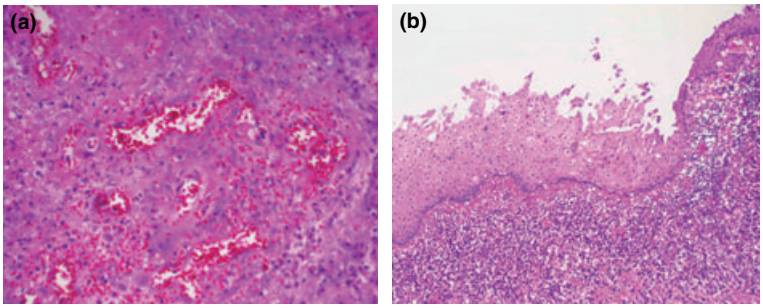

Figure 1 Histological examination of periapical lesions using paraffin sections with haematoxylin–eosin stains: (a) periapical granulomas that were rich in blood vessels, (b) radicular cysts with epithelium-lined cavity. Original magnification is ×100 (a) or ×50 (b).

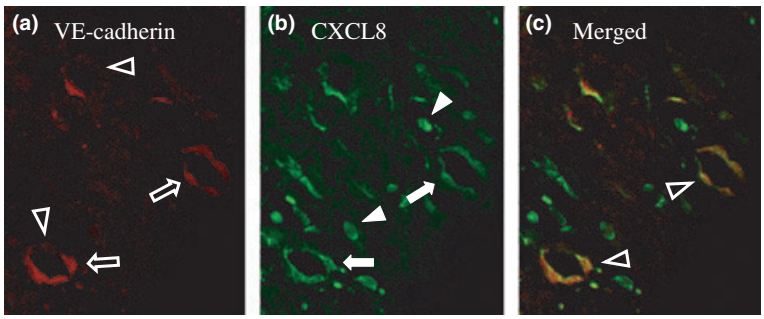

Figure 2 Two-colour immunofluorescent stains using anti-vascular endothelial (VE)-cadherin and anti-CXCL8 antibodies: (a) VE-cadherin-positive (open arrows) and -negative (open arrowheads) endothelial cells (ECs). (b) CXCL8-expressing ECs (solid arrows) and mononuclear cells (solid arrowheads). (c) Co-expression of VE-cadherin and CXCL8 by ECs (open arrowheads). Original magnification: ×400.

shown). Confocal laser scanning microscopy revealed that the ECs were strongly immunostained by the anti-VE-cadherin antibody (Figs 2a and 3a), whereas no other cells were immunostained. Negative controls using a normal rabbit IgG antibody never exhibited positive immunostaining (data not shown). The cells expressing CXCL8 or CXCL10 were predominantly ECs and mononuclear cells, as determined morphologically (Figs 2b and 3b, respectively). The composition of the mononuclear cells was not defined in this study.

As shown in Table 1, quantitative analysis was also performed by counting immunoreactive ECs. The percentages of VE-cadherin-, CXCL8-, and CXCL10-positive ECs were 40.4 ± 10.5, 60.4 ± 13.4 and 67.2 ± 13.9%, respectively. Statistical analysis showed that the percentage of VE-cadherin-positive ECs was

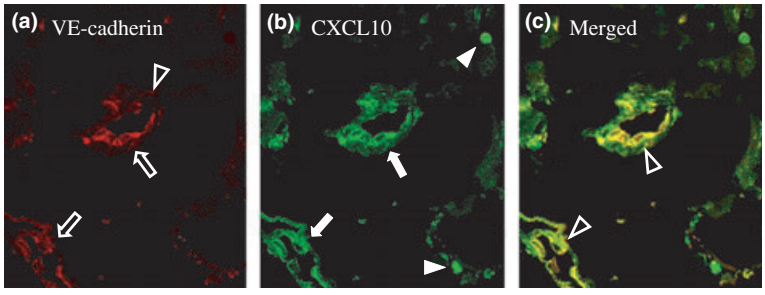

Figure 3 Two-colour immunofluorescent stains using anti-vascular endothelial-cadherin and anti-CXCL10 antibodies: (a) VE-cadherin-positive (open arrows) and -negative (open arrowheads) endothelial cells (ECs). (b) CXCL10-expressing ECs (solid arrows) and mononuclear cells (solid arrowheads). (c) Co-expression of VE-cadherin and CXCL10 by ECs (open arrowheads). Original magnification: ×400.

Table 1 VE-cadherin-, CXCL8- and/or CXCL10-expressing ECs

VE-cadherin	40.4 ± 10.5*
CXCL8	60.4 ± 13.4*
CXCL10	67.2 ± 13.9*
Co-expression	
VE-cadherin and CXCL8	37.4 ± 14.1
VE-cadherin and CXCL10	39.1 ± 13.8

VE, vascular endothelial; EC, endothelial cells.
Percentages of positive cells were shown in mean ± SD (%).
*Comparisons were considered significant at $P < 0.01$.
Differences amongst co-expression of VE-cadherin/CXCL8 and VE-cadherin/CXCL10 were not significant ($P > 0.05$).

significantly lower than the percentage of CXCL8- or CXCL10-positive ECs. Two-colour immunofluorescence staining demonstrated that 37.4 ± 14.1% of ECs expressed both VE-cadherin and CXCL8 (Fig. 2c), whereas 39.1 ± 13.8% of ECs expressed both VE-cadherin and CXCL10 (Fig. 3c). This difference was not statistically significant.

Discussion

Recruitment of inflammatory cells from the circulation in periapical lesions and the subsequent migration of these cells are important steps in the pathogenesis of chronic periapical periodontitis. This study focused on the association between transmigration and inflammation in periapical granulomas, and the expression of VE-cadherin and nitric oxide, which is known as inflammatory mediators and induces substantial tissue damages or bone resorption has been examined (Hama *et al.* 2006). Two-colour immunofluorescent microscopy for VE-cadherin and inducible nitric oxide synthase (iNOS) has demonstrated that ECs in periapical granulomas express VE-cadherin and iNOS simultaneously. In addition, iNOS-expressing macrophages and lymphocytes were present around blood vessels. Therefore, it is possible that the iNOS-expressing cells could affect VE-cadherin expression by ECs in periapical granulomas.

Based on previous findings (Hama *et al.* 2006), it was hypothesized that ECs may play a central role in modulating inflammation in periapical granulomas by controlling inflammatory cell migration. In particular, the mechanisms of cell migration from blood vessels and leukocyte chemotaxis into the site of inflammation remain to be clarified. To elucidate the hypothesis, it is essential to perform both *in vivo* and *ex vivo* experiments to determine the role of ECs in periapical granulomas. For *ex vivo* experiments, ECs such as human umbilical vein ECs are used to determine the functions of ECs (Nwariaku *et al.* 2004, Kooistra *et al.* 2005). However, these culture experiments require a background based on *in vivo* studies. Therefore, prior to laboratory experiments, human tissue samples must be analysed to ascertain whether ECs have the capability of expressing EC-related chemokines and VE-cadherin, which is a homophilic adhesion molecule localized in endothelial adherens junctions and regulates vascular permeability of the endothelial monolayer (Corada *et al.* 2001, Hermant *et al.* 2003).

Confocal microscopy revealed that 40.4 ± 10.5% of ECs in periapical granulomas were immunoreactive to an anti-VE-cadherin antibody. The percentages of VE-cadherin-positive ECs were low, however the results might be suggesting that ECs in periapical granulomas demonstrated inflammatory reaction that was accompanied by an increase in vascular permeability. Del Maschio *et al.* (1996) have shown that VE-cadherin staining was strongly reduced when PMNs adhered to TNF-treated ECs *ex vivo*. An analysis of VE-cadherin distribution has also revealed a focal loss of VE-cadherin immunostaining at sites of transmigration of $CD34^+$ cells (van Buul *et al.* 2002). Therefore, it is possible that not all ECs are associated with the maintenance of endothelium integrity during inflammation. The present data suggest that VE-cadherin-related partial vascular permeabilization of the endothelial monolayer may occur in periapical granulomas. By modulating its intercellular junctions, the endothelium may allow the transmigration of various plasma constituents and regulate the movement of circulating cells between blood vessels and inflamed areas.

Vascular endothelial-cadherin promotes cell aggregation, motility and growth (Martin *et al.* 2001), and it has been demonstrated to play a role in leukocyte adhesion-induced signalling (Saito *et al.* 1998). It has been demonstrated previously that various types of inflammatory cells express inflammatory mediators, such as nitric oxide or receptors for advanced glycation end products (RAGE) in periapical lesions (Hama *et al.* 2007) and could augment the functions of ECs (Takeichi *et al.* 1999). Therefore, inflammatory cell infiltration could be implicated in VE-cadherin expression in ECs with the stimulation of various inflammatory mediators, not only cytokines and growth factors but also nitric oxide and RAGE.

Chemokine CXCL8 and CXCL10 expression was seen in 60.4 ± 13.4 and 67.2 ± 13.9% of ECs, respectively. As expected, the number of ECs expressing CXCL8 or CXCL10 was significantly higher than the number

expressing VE-cadherin. Probably, the permeability of EC monolayers could increase and leukocyte chemotaxis mediated by CXCL8 and CXCL10 might occur in periapical granulomas. To elucidate these hypotheses, *ex vivo* experiments will be performed. Not only ECs, but also the other infiltrating cells expressed these CXC chemokines. Morphological examinations in this study did not determine the cellular composition of the CXCL8- or CXCL10-expressing cell population. However, the CXCL8-expressing cells might be macrophages and T-lymphocytes (Bodet *et al.* 2005), whereas the CXCL10-expressing cells might be PMNs and fibroblasts (Simpson *et al.* 2000).

The cell–cell contact of ECs and leukocytes in the bloodstream might be important in regulating neovascularization in vascular inflammation (Engelhardt and Wolburg 2004) and the additional leukocyte chemotaxis into inflamed areas. In this study, it was demonstrated that some ECs co-expressed VE-cadherin and CXCL8 or CXCL10. Therefore, ECs may have a capability to control vascular permeability and augment leukocyte chemotaxis.

Conclusions

Vascular endothileal-cadherin and CXCL8 or CXCL10 expressed by ECs could be important for inflammatory cell recruitment in periapical granulomas. ECs might play a central role in inflammation and immune cell responses by controlling leukocyte migration and chemotaxis.

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