Co-production of vascular endothelial cadherin and inducible nitric oxide synthase by endothelial cells in periapical granuloma

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Abstract

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Aim To clarify the mechanisms of inflammatory cell migration in human periapical granulomas by examining vascular endothelial (VE) cadherin and inducible nitric oxide synthase (iNOS)-producing cells.

Methodology Periapical tissues were obtained from patients during endodontic surgery and were divided into two portions. After fixing the tissues with acetone or 4% paraformaldehyde in phosphate-buffered saline, 5-μm-thick paraffin or cryostat sections were prepared, respectively. The paraffin sections of the inflamed tissues were evaluated histologically with haematoxylin–eosin stains. Cryostat sections of the tissue, diagnosed as periapical granulomas, were then examined by either immunohistochemistry using anti-human VE-cadherin or iNOS antibodies (Abs) for the characterization of infiltrating cells. In addition, co-localization of VE-cadherin and iNOS production was also analysed by two-colour immunofluorescence image analysis.

Results Endothelial cells were strongly stained with iNOS Abs. Macrophages, lymphocytes, polymorphonuclear leucocytes and fibroblasts also exhibited iNOS production. These iNOS-positive cells accumulated around the blood vessels. On the other hand, VE-cadherin production was exhibited in only endothelial cells. Two-colour immunofluorescence image analysis using VE-cadherin and iNOS Abs demonstrated that iNOS-producing endothelial cells also showed VE-cadherin production.

Conclusions Vascular endothelial-cadherin produced by endothelial cells could be regulated by iNOS-producing cells in periapical granulomas and might play a pivotal role in vascular permeability.

Keywords: endothelial cells, immunohistochemistry, inducible nitric oxide synthase, periapical granuloma, VE-cadherin.

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Introduction

Apical periodontitis is a chronic inflammatory condition caused by bacterial infection (Takahashi 1998). After microbial invasion of periapical tissues, both nonspecific inflammatory and specific immunologic responses persist in the host tissues. Lipopolysaccharide (LPS), which forms an integral part of the outer layer of gram-negative cell walls, can egress through the apical foramen into the periapex to initiate and sustain apical periodontitis. Both nonsurgical and surgical endodontic treatments are essentially debridement procedures to remove microorganisms. The structural components of periapical lesions depend on the balance between the microbial factors and the host defences. Many

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investigators have examined microorganisms in root canals (Haapasalo 1989, Yanagiguchi *et al.* 1995); however, not only bacterial antigenicity, but also host responses must be characterized.

Nitric oxide has been identified as an endothelial-derived relaxing factor, which is associated with blood pressure (Furchgott & Zawadzki 1980); however, it is also known as an inorganic and reactive radical gas. Nitric oxide has a variety of biological activities including the induction of apoptosis (Sagoo *et al.* 2004) and intracellular iron loss (Hibbs *et al.* 1987), as well as the inhibition of mitochondrial respiration (Stuehr & Nathan 1989) and DNA synthesis (Lepoivre *et al.* 1992). Thus, it is thought to modulate inflammation and to induce substantial tissue damage or metabolic dysfunction. In relation to these findings, Di Maio *et al.* (2004) demonstrated that healthy pulp tissues failed to exhibit an inducible form of nitric oxide synthase (iNOS), whereas acute inflammation enhanced the mRNA and protein levels of iNOS. Therefore, iNOS may have a role in inflammatory pathological processes.

Inducible NOS has been shown to produce a large amount of nitric oxide with a stimulation of bacterial LPS and inflammatory cytokines such as interferon-γ (IFN-γ), interleukin (IL)-1β or tumour necrosis factor α (Breft *et al.* 1991, Xie *et al.* 1992). The association of iNOS and the inflammatory cytokines in periapical lesions has been examined (Takeichi *et al.* 1998, 1999). Endothelial cells, macrophages, lymphocytes and fibroblasts in periapical lesions demonstrated the production of iNOS with the association of these cytokines. Thus, the release of nitric oxide could be largely controlled by inflammatory cytokines with local mechanisms responding to the stimulation in periapical lesions.

On the other hand, the functions of nitric oxide in periapical lesions have not been clearly determined yet. Endothelial cells showed marked production of iNOS in periapical lesions (Takeichi *et al.* 1998, 1999), and could be a potent regulator of inflammation in periapical lesions. Endothelial cells constitute an important interface lining the internal vascular surface, and regulate the passage of solutes and circulating cells from blood to tissues (Engelhardt & Wolburg 2004). In the present study, endothelial cells were focused upon to determine the mechanisms of cell migration with the association of iNOS.

Endothelial cell junctions regulate barrier functions of the vascular endothelium that participates in vascular permeability to plasma proteins and circulating cells. The endothelium expresses a cell-specific member of the cadherin family, known as cadherin-5 or vascular endothelial (VE)-cadherin (Telo *et al.* 1998). VE-cadherin is essential for endothelial functions and could be a key molecule of vascular permeability (Corada *et al.* 2001).

To clarify the mechanisms of inflammatory cell migration in periapical granulomas, VE-cadherin- and iNOS-producing cells were examined by immunohistochemistry; the interactions between VE-cadherin- and iNOS-producing cells were determined by two-colour immunofluorescence image analysis.

Materials and methods

Patients

In patients having apical periodontitis based on the clinical criteria (size of radiolucency around the apex, percussion pain, swelling of mucosa around the apex of a tooth and fistulas), 31 patients (16 males and 15 females) ranging in age from 19 to 79 years were examined. No systemic disease was observed in any patients, and antibiotics had not been taken during the previous 6 months. This study was approved by the ethics committee in Nihon University School of Dentistry, based on the Declaration of Helsinki. Written consent was obtained from all patients prior to the collection of samples.

Preparation of samples

Tissues around periapical lesions were obtained from patients at the time of surgical treatment (apicoectomy) for the removal of periapical lesions. Immediately, the tissues were sectioned into two portions of similar size. One portion was fixed with acetone and then embedded in paraffin. Another portion was fixed with 4% paraformaldehyde in phosphate-buffered saline (PBS) and then embedded in a compound (OCT compound®; Tissue-Tek, Elkhart, IN, USA) to freeze at optimum cutting temperature, followed by freezing in dry ice-acetone. Five micrometre-thick paraffin and cryostat sections were then prepared, respectively. The serial sections were examined.

Histological examination of samples

Sectioned areas of embedded samples were randomly selected, and at least three different places in one sample block were examined. Histological examination

was performed using serial sections of surgically removed periapical lesions. Paraffin sections were deparaffinized with xylene, and re-hydrated with graded ethanol, and were then subjected to haematoxylin–eosin stains. The specimens were assessed histologically under a light microscope.

Immunohistochemical analysis

To examine human VE-cadherin and human iNOS-producing cells in periapical granulomas, immunohistochemistry was performed for cryostat sections using the avidin–biotinylated enzyme complex method. In brief, the sections were incubated with 0.3% H_2O_2 in methanol for 30 min to quench endogenous peroxidase, and then nonspecific binding was blocked with 10% normal horse serum (Vector Laboratories, Inc., Burlingame, CA, USA) for 20 min. The sections were incubated for 2 h with appropriate dilution of anti-human VE-cadherin monoclonal antibodies (Abs) (Chemicon international, Temecula, CA, USA). After rinsing the sections three times with PBS, biotinylated horse anti-mouse IgG Abs (×200) were incubated for 30 min. The subjects were then rinsed three times with PBS and incubated with avidin–biotin peroxidase complex (Vector Laboratories, Inc.) for 30 min. After a final wash with PBS, optimum colour development of VE-cadherin-positive cells was achieved with horseradish peroxidase substrates (3,3′-diaminobenzidine; Vector Laboratories, Inc.) for 10 min. Counter stain was performed using methyl green. The specimens were mounted and VE-cadherin-positive cells were carefully examined using a light microscope.

For the detection of iNOS-producing cells, similar methods using anti-human iNOS monoclonal Abs (R&D systems, Minneapolis, MN, USA) were performed. Biotinylated anti-mouse IgG Abs were then incubated, followed by the incubation of avidin–biotin glucose oxidase complex. For the final colour development of iNOS-positive cells, glucose oxidase substrates (tetranitroblue tetrazolium; Vector Laboratories, Inc.) were used. Normal rabbit IgG Abs (Cayman chemical, Ann Arbor, MI, USA) were incubated for a negative control in place of VE-cadherin or iNOS Abs.

Two-colour immunofluorescence image analysis

The interactions of VE-cadherin- and iNOS-producing cells in periapical granulomas were analysed by two-colour immunofluorescence image analysis using human VE-cadherin and iNOS Abs. In brief, the samples were blocked with normal rabbit serum (Cederlane Laboratories Ltd, Hornby, ON, Canada) for 20 min and then co-incubated with goat anti-human VE-cadherin polyclonal Abs (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) and anti-human iNOS monoclonal Abs for 2 h. After washing the sections, rhodamine isothiocyanatel (RITC)-conjugated rabbit anti-goat IgG Abs (Open Biosystems, Huntsville, AL, USA) and fluorescein isothiocyanatel (FITC)-conjugated rabbit anti-mouse IgG Abs (P.A.R.I.S., Compiègne, France) were incubated for the detection of VE-cadherin and iNOS, respectively. The specimens were mounted with aqueous mounting medium (fluoromount-G; Southern Biotechnology Associates, Birmingham, AL, USA). VE-cadherin and iNOS positive cells were then carefully examined using a fluorescent microscope (Model BHF-142; Olympus, Tokyo, Japan).

Results

Histological evaluation of periapical lesions

For the definition of pathological features of periapical lesions, paraffin sections of periapical lesions ($n = 31$) were evaluated histologically by haematoxylin–eosin stains. The definition of radicular cysts by Nair (1998) was referred to evaluate periapical lesions. Of 31 periapical lesions examined by a light microscope, 27 subjects exhibited granulomatous lesions, in which a large number of inflammatory cells were infiltrated (Fig. 1a). No epithelial cells were observed in any views of these samples, and the specimens were diagnosed as periapical granulomas. Another four lesions exhibited granulomatous tissues with complete epithelial lining and surrounding collagen fibres (Fig. 1b). These specimens were, therefore, diagnosed as radicular cysts and were excluded from this study.

Immunohistochemical and immunofluorescence microscopic analyses

In order to examine the interactions of VE-cadherin and iNOS in periapical granulomas, the cryostat sections ($n = 27$) were analysed using VE-cadherin and iNOS monoclonal Abs. Types of positive cells were determined morphologically under a light microscope. As a result, endothelial cells strongly reacted against iNOS monoclonal Abs (Fig. 2a). iNOS-producing cells were present around blood vessels. Macrophages, lymphocytes and plasma cells exhibited immunoreactivity to iNOS (Fig. 2b). Polymorphonuclear leucocytes (PMNs) also showed iNOS-positive staining; however, the

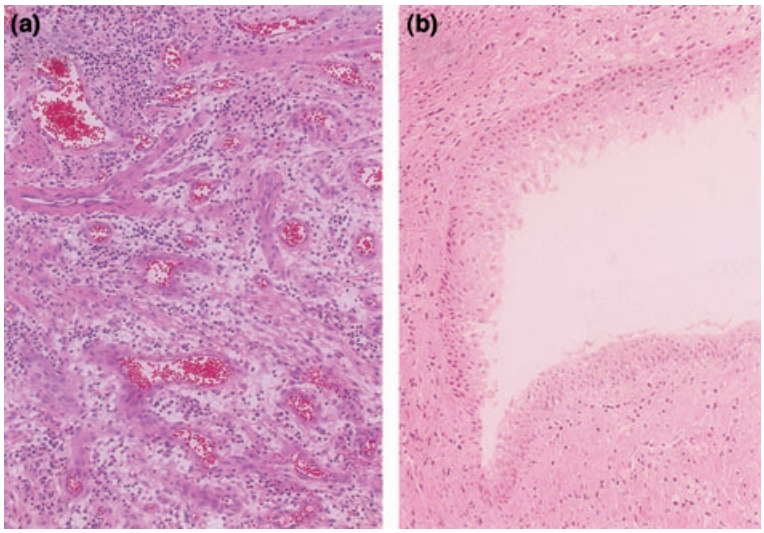

Figure 1 Histological evaluation of periapical lesions by haematoxylin–eosin stains: (a) periapical granulomas showing granulomatous tissues without epithelial cells, (b) radicular cysts with epithelial cells. Original magnification is ×200 (a) or ×100 (b).

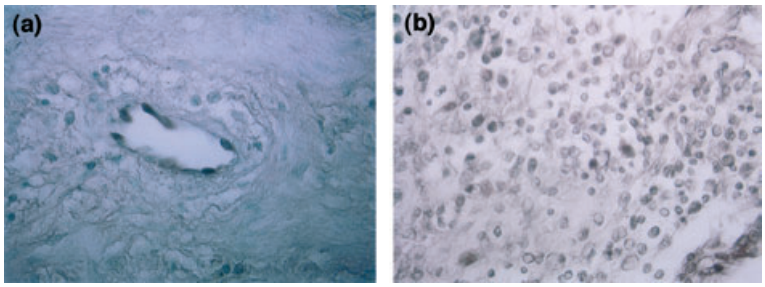

Figure 2 Immunohistochemical analysis of periapical granulomas using human iNOS Abs: (a) endothelial cells showing iNOS production, (b) mononuclear cells and PMNs showing iNOS production. Original magnification is ×200.

intensity was lower than that of mononuclear cells. On the other hand, VE-cadherin immunohistochemistry demonstrated that the reactivity of VE-cadherin Abs was obvious in endothelial cells, but not in mononuclear cells, PMNs and fibroblasts (Fig. 3). Two-colour immunofluorescence image analysis demonstrated that VE-cadherin-producing endothelial cells were also positive for iNOS immunoreactivity (Fig. 4c,d). In addition, iNOS-producing mononuclear cells accumulated around the blood vessels. Negative controls without both Abs did not show positive stains (Fig. 4a,b).

Figure 3 Immunohistochemical analysis of periapical granulomas using human VE-cadherin Abs. Endothelial cells were positive for VE-cadherin. Original magnification is ×200.

Discussion

Nitric oxide is a multifunctional mediator and participates in inflammatory tissue destruction (Van't Hof & Ralston 2001). Nitric oxide has also been shown to be implicated in the osteoblast activation and osteoclast differentiation, which are associated with bone remodelling. The association of nitric oxide in periapical granulomas and radicular cysts has been examined in order to elicit the mechanism of bone resorption in periapical lesions (Takeichi *et al.* 1998, 1999). It has been speculated that inflammatory cytokines such as IL-1β and IFN-γ synergistically up-regulate iNOS production in periapical granulomas (Takeichi *et al.* 1998). On the other hand, cytokines share many of its biological activities with the other cytokines (Le & Vilcek 1987),

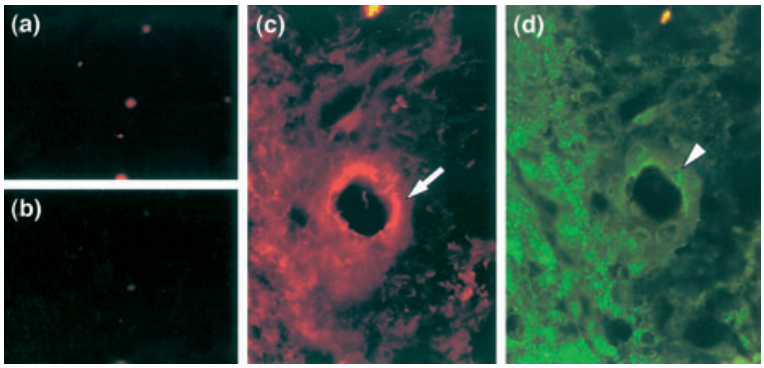

Figure 4 Two-colour immunofluorescence image analysis using human VE-cadherin and iNOS Abs: (a) negative controls without VE-cadherin Abs, (b) negative controls without iNOS Abs, (c) VE-cadherin-positive endothelial cells: arrow, (d) iNOS-positive endothelial cells and mononuclear cells: arrow head. Original magnification is ×400.

and acts in a network of factors directing the inflammatory reaction. On the basis of the present and previous findings, it is suggested that nitric oxide plays a pivotal role in regulating inflammatory reaction in periapical lesions with the association of cytokines.

However, more detailed mechanisms of how nitric oxide is related to periapical lesions are unknown. In this study, endothelial cell junctions were focused upon, which are considered to be responsible for several of the structural and functional characteristics specific to leucocytes. This property is important in immune systems, because regulated leucocyte adhesion is critical to immunity and inflammation, and also controls cellular positioning, cell–cell interactions and immune cell responses (van Buul *et al.* 2002).

In an attempt to determine the characteristics of endothelial cell junctions in periapical lesions, periapical granulomas were analysed for the production of VE-cadherin with the association of iNOS. VE-cadherin and iNOS production in periapical granulomas was examined initially. Immunohistochemistry using the cryostat sections demonstrated that endothelial cells reacted against VE-cadherin-specific Abs. On the other hand, iNOS immunohistochemistry exhibited that endothelial cells showed strong immunoreactivity against iNOS Abs as well as macrophages, rather than the other mononuclear cells or PMNs. iNOS-producing cells accumulated around the blood vessels, suggesting that these inflammatory cells are associated with endothelial cells. Probably, the inflammatory cells are stimulated with biologically active factors derived from endothelial cells. As a result, iNOS could be spontaneously synthesized through cell–cell communication by these cells. This iNOS production could augment the functions of endothelial cells. Based on these findings, it was hypothesized that iNOS produced by endothelial cells or the other inflammatory cells could be associated with VE-cadherin production by endothelial cells.

In order to elucidate the hypothesis described above, analysis of the same specimens with additional immunohistochemical analysis using both VE-cadherin and iNOS Abs was undertaken. Two-colour immunofluorescence image analysis clearly demonstrated that VE-cadherin-producing endothelial cells also have a capability to produce iNOS. The data indicated that endothelial cells could be controlled by autocrine or paracrine of nitric oxide for VE-cadherin production in periapical granulomas. Synergistic effects of VE-cadherin and nitric oxide might also be important in controlling cellular infiltration to the site of inflammation. Taken together, periapical granulomas can be regulated and delicately balanced with the biological activities of nitric oxide with respect to VE-cadherin production derived from endothelial cells. These findings may contribute to the understanding of periapical granulomas.

The prevention of nitric oxide synthesis would in turn attenuate the down-regulation of VE-cadherin (González *et al.* 2003). The inhibition of VE-cadherin expression causes disorganization of endothelial junctions and the following vascular permeability, which induces the enhancement of inflammatory cell migration (Maschio *et al.* 1996). As a result, inflammation could be increased at inflamed areas.

The amino acid L-arginine has been shown to be the precursor for the synthesis of nitric oxide, and endothelial cells cultured in the absence of L-arginine for 24 h showed a decrease in the release of nitric oxide (Palmer *et al.* 1988). This reaction would be restored by L- but not D-arginine (Billiar *et al.* 1992), suggesting that the conversion of L-arginine to nitric oxide is specific. Thus, L-arginine analogue could be used as an inhibitor of the generation of nitric oxide in stimulated inflammatory cells. Recently, it has been shown that the onset of experimentally induced colitis in mice is blocked by ONO-1714 (Naito *et al.* 2001). The

ONO-1714 is 875 times more active than L-arginine analogue, N^G-monomethyl-L-arginine (L-NMMA) and 34 times more selective for iNOS. Therefore, it was hypothesized that iNOS-specific inhibitors such as ONO-1714 could be used as a pharmacological agent in the management of periapical lesions.

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