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Inhibition of nuclear factor- κ B prevents the development of experimental periapical lesions

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Abstract

Introduction: Nuclear factor-kappa B (NF- κ B) is an important transcriptional regulator of angiogenesis involving B-cell lymphoma-2 (Bcl-2) and Bcl-2-associated X protein (Bax) signaling pathways. Thus, inhibition of NF- κ B may suppress the development of periapical lesions via blockage of angiogenesis. Accordingly, we examined the effects of NF- κ B decoy oligodeoxynucleotide (ODN) treatment on experimentally-induced periapical lesions.

Methods: Periapical lesions were induced in the mandibular first molars of 5-week-old male Wistar rats by the application of lipopolysaccharide to the pulp. NF- κ B decoy ODN or NF- κ B decoy scramble (control) was injected intraperitoneally every 7 days, starting 1 day before pulp exposure. After 28 days, samples were retrieved, and digital radiographs were taken for radiomorphometry. Samples were processed for (1) immunohistochemistry of CD31, Bcl-2, and Bax; (2) laser capture microdissection to analyze *Bcl-2*, *Bax*, *chemokine (C-X-C motif) ligand 1* (*CXCL1*), *CXC receptor 2* (*CXCR2*) and vascular endothelial cell growth factor receptor 2 (*VEGFR2*) mRNA expression in CD31+ endothelial cells; (3) enzyme-linked immunosorbent assay to determine NF- κ B/p65 activity; and (4) western blotting for VEGF expression.

Results: NF- κ B decoy ODN treatment significantly reduced lesion size, NF- κ B/p65 activity, and the density of CD31+ endothelial cells in the lesion. NF- κ B decoy ODNs also downregulated *CXCL1*, *CXCR2*, and *VEGFR2* mRNAs and upregulated *Bax* mRNA in endothelial cells but did

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not affect *Bcl2* mRNA in endothelial cells. VEGF protein expression in the lesions was significantly decreased.

Conclusion: Inhibition of NF- κ B activity by decoy ODN treatment suppressed the development of experimentally-induced periapical lesions with a concomitant reduction in angiogenic responses in endothelial cells.

Keywords

B-cell lymphoma-2; Bcl-2-associated X protein; nuclear factor- κ B; periapical lesion; vascular endothelial growth factor

Introduction

Apical periodontitis is a common oral inflammatory disease caused by bacterial infection in the root canal system and is characterized by the formation of granulation tissues with concomitant bone resorption in the periradicular region (1, 2). This disease involves complex cellular and molecular mechanisms where various bioactive molecules, such as cytokines, lytic enzymes, and growth factors, contribute to tissue destruction (1, 2).

Angiogenesis is the growth of new blood vessels from pre-existing vasculature and is essential for several pathological and physiological processes, including granulation tissue development and wound healing (3). Abnormal or excessive angiogenesis is a characteristic of several disorders, such as cancer (4) and chronic inflammation (5). Angiogenesis is mediated by several cytokines and chemokines including interleukin-1 (IL-1), IL-8, and tumor necrosis factor- α (6). Moreover, angiogenic factors, such as fibroblast growth factor (7) and vascular endothelial cell growth factor (VEGF) (7), have been detected in periapical lesions. In experimentally induced rat periapical lesions, an increase in microvascular density and upregulation of mRNAs encoding angiogenic factors, such as VEGF receptor 2 (VEGFR2), B-cell lymphoma-2 (Bcl-2; a prosurvival and pro-angiogenic signaling molecule), chemokine (C-X-C motif) ligand 1 (CXCL1; a pro-angiogenic chemokine) and CXC receptor-2 (CXCR2), in endothelial cells occurs coincidently with the expanding phase of the lesion (8). These findings suggest these angiogenic factors have critical roles in the pathogenesis of apical periodontitis.

Nuclear factor-kappa B (NF- κ B) is activated by various neurotoxic and apoptotic stimuli (9). NF- κ B induces the expression of several pro-apoptotic molecules, such as Bcl-2-associated X protein (Bax) and Bcl-2 (10). Apoptosis is induced by upregulation of Bax, activation of caspases, and downregulation of Bcl-2 (11). Furthermore, NF- κ B is a critical factor in angiogenesis, particularly in oncology (12). For example, tumor cell-derived VEGF induces Bcl-2 expression in endothelial cells, which in turn activates NF- κ B and upregulates the expression of pro-angiogenic chemokines CXCL1 and CXCL8 (13). Thus, inhibition of NF- κ B in the angiogenic pathway may suppress angiogenesis and reduce periapical lesions (14).

Recently developed gene therapies include decoy oligonucleotides (ONs) and oligodeoxynucleotides (ODNs) (15). Decoy ONs are short synthetic fragments of DNA or RNA, which mimic complementary nucleic acid sequences to prevent transcription factor

binding to target gene promoter regions. Because of target recognition specificity, off-target effects are limited (16). ODNs are decoys for specific transcription factors that attenuate authentic *cis-trans* interactions and facilitate removal of *trans*-factors from endogenous *cis*-elements, thereby modulating gene expression.

We hypothesized that inhibition of NF- κ B suppresses periapical lesion development via blockage of angiogenesis. In this study, we examined the effects of NF- κ B decoy ODN treatment on experimentally induced periapical lesions.

Materials and Methods

Animal preparation

All rats were used in compliance with the guidelines of Niigata University Intramural Animal Use and Care Committee. Five-week-old male Wistar rats ($n = 24$) were anesthetized by an intraperitoneal injection of 8% chloral hydrate (350 mg/kg). Pulp exposure was performed at the occlusal surface of both sides of the mandibular first molars, and 1 μ L lipopolysaccharide (from *Escherichia coli* 0111:B4; Sigma, St. Louis, MO; 10 mg/mL) was applied to the pulp with sterile paper points. Pulp cavities were sealed with a temporary filling material (Cavition, GC, Tokyo, Japan). In the experimental group of animals ($n = 12$), 2 mL saline containing ribbon-type NF- κ B decoy ODN (NF- κ B decoy; Gene Design, Inc., Osaka, Japan) was injected intraperitoneally 1 day before pulp exposure and on days 6, 13, 20, and 27 after pulp exposure (50 nmol/week). In control animals ($n = 12$), ribbon-type NF- κ B decoy scramble (50 nmol/week; Gene Design, Inc.) was administered in a similar manner.

Sample preparation

On day 28 after lesion induction, animals were sacrificed by transcardiac perfusion of periodate lysine paraformaldehyde fixative, except for animals analyzed by western blotting. Both sides of the mandibular first molars were retrieved with the surrounding bones and soaked in the same fixative for 24 h.

Eight rats were used for immunohistochemistry and enzyme-linked immunosorbent assay (ELISA). Immediately after retrieval of the right mandibles ($n = 4$ in each group), digital radiographs were taken as described below. The specimens were demineralized with 10% ethylenediaminetetraacetic acid (EDTA), embedded as frozen blocks, and sliced in 8- μ m sections (CM1900; Leica, Wetzlar, Germany) for immunohistochemistry.

For laser capture microdissection (LCM; $n = 8$ rats), the right first molars ($n = 4$ in each group) were retrieved and demineralized with a mixture of 10% EDTA and storage medium (RNAlater; Thermo Fisher Scientific, Waltham, MA). Frozen samples were cut into 30- μ m sections and mounted on glass slides with a PEN foil membrane for LCM (Leica Microsystems). Four serial sections with the root apex were selected and used for LCM.

For western blot analysis, rats ($n = 8$) were sacrificed by intraperitoneal injection of an overdose of 8% chloral hydrate. The right first molars ($n = 4$ in each group) were retrieved and demineralized with a mixture of 10% EDTA and storage medium (RNAlater; Thermo

Fisher Scientific). Frozen samples were cut into 30- μ m sections and mounted on glass slides for LCM (Leica Microsystems). Four serial sections with the root apex were selected and used for western blot analysis.

Digital morphometric analysis

Digital radiographs of the right mandibles in both groups ($n = 8$) were taken with image capture in a digital radiograph unit (Venus, Yoshida Seikou, LTD., Trophy Windows, Trophy Radiologie Japan), and the size of the radiolucent area around the distal root was measured using ImageJ software (Version 1.37v; National Institutes of Health, Bethesda, MD).

ELISA for NF- κ B p65 activity

NF- κ B activity was assessed by the level of p65 in the nuclear fraction using the NF- κ B/p65 ActivELISA™ kit (Imgenex, San Diego, CA). Nuclear extracts were prepared and subjected to a sandwich ELISA according to the manufacturer's protocol. The optical density of samples was determined using an ELISA microplate reader (Infinite F50, Tecan, Mannedorf, Switzerland) at 450 nm.

Immunohistochemistry

Immunoperoxidase staining was performed with monoclonal anti-CD31 antibodies (a general endothelial cell marker: Serotec, Oxford, UK; diluted 1:100), polyclonal anti-Bcl-2 antibodies (Millipore; diluted 1:2000), or polyclonal anti-Bax antibodies (Novus Biologicals; diluted 1:300) as previously described (8). Secondary antibodies were biotinylated horse anti-mouse IgG (Vector Laboratories, Burlingame, CA) for anti-CD31 and biotinylated goat anti-rabbit IgG (Vector Laboratories) for anti-Bcl-2 and anti-Bax. Immunoreactivity was visualized with a DAB Substrate Kit (Vector Laboratories). Negative control staining was performed in parallel by incubating sections with phosphate-buffered saline instead of primary antibody.

For quantitative analysis, five randomly selected fields (200 μ m $\times$ 100 μ m) from a representative section per sample were displayed on a monitor connected to a light microscope (Nikon) at 40 $\times$ magnification (objective lens), as previously described (17). The immunostained area was plotted, and pixel counts were determined using ImageJ software (Version 1.37v; National Institute of Health). The percentage of the immunostained area in the total area and Bax/Bcl-2 ratios were calculated.

LCM and real-time polymerase chain reaction (PCR)

Immunoperoxidase staining for CD31 was performed as described above. Cell collection was performed with an LCM microscope (Leica AS LMD; Leica) with a pulsed 337-nm UV laser. Approximately 200 CD31+ endothelial cells in periapical lesions were retrieved as previously described (18). Total RNA was extracted and purified ($n = 4$) as previously described (19). First-strand cDNA synthesis was performed with TaqMan reverse transcription reagents (Applied Biosystems, Carlsbad, CA). Probe and primer sets for TaqMan Gene Expression Assays (Rn99999125_m1; *Bcl-2*, Rn02532082_g1; *Bax* [*rBax alpha* mRNA], Rn00578225_m1; *CXCL1*, Rn02130551_s1; *CXCR2* [*interleukin 8 receptor beta*], Rn00564986_m1; *KDR* [*VEGFR2*]; and Rn99999916_s1; *glyceraldehyde-3-*

phosphate dehydrogenase [*GAPDH*]) were obtained from Applied Biosystems. Total RNA (0.01 µg/30 µL reaction mixture) was prepared using TaqMan Universal PCR Master Mix (Applied Biosystems). The reactions were incubated in a 48-well clear optical reaction plates using a StepOne Sequence Detection System (Applied Biosystems), and the data were normalized by the data for *GAPDH* as a control.

Western blotting

The periapical lesion area was retrieved from the glass slides using a microsurgery knife under a stereomicroscope. Total protein was extracted from the retrieved periapical lesion samples (n = 4 teeth in each experimental group), subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis, transferred to membranes, probed with primary antibodies against VEGF (R&D Systems, Minneapolis, MN) or GAPDH (GeneTex, Irvine, CA), and then probed with peroxidase-coupled secondary antibodies. Protein signals were visualized using an enhanced chemiluminescence system (GE Healthcare, Pittsburgh, PA), and protein expression was calculated relative to that of GAPDH.

Statistical analysis

Data were analyzed by Mann-Whitney *U*-tests. Differences with *P* values of less than 0.05 were considered significant.

Results

NF-κB decoy ODN treatment suppressed periapical lesion development, NF-κB activity, and CD31 immunoreactivity

Radiographs from both the NF-κB decoy ODN-treated and NF-κB decoy scramble-treated groups showed periapical radiolucency in the first molar (Fig. 1a, b). In both groups, the pulp showed total necrosis and periapical lesions composed of a mass of granulation tissue with apical abscesses was developed (Fig. 1c, d). The lesion size in the NF-κB decoy ODN-treated group was significantly smaller than that in the NF-κB decoy scramble-treated group (Fig. 1e).

CD31+ endothelial cells were observed in all experimental groups (Fig. 2a, b). The CD31+ area in the NF-κB decoy ODN-treated group was significantly smaller than in the NF-κB decoy scramble-treated group (Fig. 2c). NF-κB activity, assessed by measuring the levels of p65 in the nuclear fractions with ELISA, was significantly lower in the NF-κB decoy ODN-treated group than that in the NF-κB decoy scramble-treated group (Fig. 2d).

NF-κB decoy ODN treatment affected the Bax/Bcl-2 ratio

Bax and Bcl-2 expression was observed in all experimental groups (Fig. 3a-d). Bcl-2 and Bax were expressed in various types of cells, including endothelial cells (Fig. 3a-d). The Bcl-2+ area in the NF-κB decoy ODN-treated group was significantly smaller than in the NF-κB decoy scramble-treated group (Fig. 3e), whereas the Bax+ area was significantly larger in the NF-κB decoy ODN-treated group than in the NF-κB decoy scramble-treated group (Fig. 3f). Thus, the Bax/Bcl-2 ratio in the NF-κB decoy ODN-treated group was significantly higher than that in the NF-κB decoy scramble-treated group (Fig. 3g).

NF- κ B decoy ODN treatment decreased VEGF expression, downregulated *CXCL1*, *CXCR2* and *VEGFR2* mRNA, and upregulated *Bax* mRNA

In western blot analysis, VEGF expression in the NF- κ B decoy ODN-treated group was downregulated compared with that in the NF- κ B decoy scramble-treated group (Fig. 4a). Although there were no significant differences in the levels of *Bcl-2* mRNA in CD31+ endothelial cells in both groups (Fig. 4b), *Bax* mRNA levels in CD31+ endothelial cells in the NF- κ B decoy ODN-treated group were upregulated compared with those in the NF- κ B decoy scramble-treated group (Fig. 4c). The *Bax/Bcl-2* mRNA ratio in the NF- κ B decoy ODN-treated group was significantly increased compared with that in the NF- κ B decoy scramble-treated group (Fig. 4d). Notably *CXCL1*, *CXCR2*, and *VEGFR2* mRNA in CD31+ endothelial cells in the NF- κ B decoy ODN-treated group was significantly downregulated compared with that in the NF- κ B decoy scramble-treated group (Fig. 4e-g).

Discussion

In this study, we found that NF- κ B decoy ODN treatment reduced the activity of NF- κ B and decreased the size of rat experimental periapical lesions 28 days after induction, when osteoclastic activity has decreased and the periapical lesion has stabilized (8). The NF- κ B p65 level in the nuclear fraction was significantly lower in the NF- κ B decoy ODN-treated group than that in the control group, indicating that NF- κ B decoy ODN reduced the nuclear translocation of NF- κ B. Moreover, the density of CD31+ endothelial cells in NF- κ B decoy ODN-treated animals was significantly decreased compared with that in NF- κ B decoy scramble-treated animals. NF- κ B inhibition increased *Bax* mRNA and decreased *CXCL1*, *CXCR2* and *VEGFR2* mRNA but did not affect the expression of *Bcl2* mRNA in endothelial cells. Moreover, VEGF protein expression was downregulated in NF- κ B decoy ODN-treated animals. These results suggested that reduction of periapical lesions by blocking NF- κ B activity was associated with inhibition of angiogenic pathways.

Bcl-2 and *Bax* are homologous proteins belonging to the *Bcl-2* family but have opposite effects on cell survival and death; *Bcl-2* prolongs cell survival, and *Bax* accelerates apoptosis (20). Expression of *Bcl-2* and *Bax* in the endothelial cells of periapical lesions was investigated to determine their roles in the regulation of angiogenesis *in vivo* through inhibition of NF- κ B. NF- κ B has a crucial role in angiogenic and apoptotic responses (21). Additionally, NF- κ B activity is important in endothelial cell survival (22), and NF- κ B inhibition increases *Bax* expression (23). Overexpression of *Bax* suppresses breast cancer growth in immunosuppressed mice (24). *Bax* mRNA was upregulated in CD31+ endothelial cells in NF- κ B decoy ODN-treated animals. In contrast, there were no significant differences in *Bcl-2* levels in both groups, and thus, the *Bax/Bcl-2* ratio increased in NF- κ B decoy ODN-treated animals. These findings are consistent with the finding that (25). NF- κ B blockade upregulates *Bax*, and subsequently increases *Bax* to *Bcl-2* ratio, and prevents the development of λ -carrageenin-induced granulation tissue in rats. (25). The decrease of *Bcl-2*-immunostained area in the NF- κ B decoy ODN-treated group (Fig. 2e) could reflect the decrease of *Bcl-2*-expressing endothelial cells, although the level of *Bcl-2* mRNA in endothelial cells was similar in both groups (Fig. 3b). Taken together, our findings suggest

that an increase in the ratio of Bax/Bcl-2 in endothelial cells is associated with NF- κ B decoy ODN-induced suppression of lesion development.

The present immunohistochemical analysis showed that Bcl-2+ area in NF- κ B decoy ODN-treated animals was significantly smaller than that in the control animals, while there was no significant difference in the level of Bcl-2 mRNA in endothelial cells. Like a previous report from bcl-2 mRNA and protein expression in hepatocellular carcinomas (26), it can be inferred that the difference between bcl-2 mRNA expression and histological pattern caused by a post-translational mechanism of bcl-2 protein degradation and indicating that bcl-2 does not play a substantial role in the progress of periapical lesion under NF- κ B decoy ODN treatment.

In angiogenesis, VEGF regulates vascular permeability and promotes the proliferation and migration of endothelial cells (27). VEGF specifically binds to and reacts with tyrosine kinase receptors, such as VEGFR1, -R2, and -R3. In particular, VEGFR2 is a major transducer of VEGF signals in endothelial cells (28). VEGF mediates endothelial cell survival by inducing Bcl-2 expression in a pathway that requires VEGF binding to VEGFR2 and activation of phosphatidylinositol 3-kinase/Akt signaling (29). The VEGFR2 expression decreases in human umbilical vein endothelial cells after morusin treatment, which indicates that morusin promotes anti-angiogenesis (30). NF- κ B is activated in endothelial cells exposed to VEGF, and if the activity of NF- κ B is blocked, the expression of CXC chemokines, such as CXCL8 and CXCL1, is downregulated (13). Thus, NF- κ B regulates the expression of CXC chemokines that have critical roles in the regulation of angiogenesis during many pathological processes (31). In this study, NF- κ B decoy ODN treatment downregulated *CXCL1*, *CXCR2* and *VEGFR2* mRNAs in endothelial cells and decreased VEGF protein expression in periapical lesions. In rats, CXCL1 and CXCR2 are related to growth-regulated oncogene/cytokine-induced neutrophil chemoattractant-1 (GRO/CINC-1), which is a rat homologue of human CXCL8 (also known as interleukin-8), a member of the CXC subfamily of chemokines (32, 33). The density of CD31+ endothelial cells was decreased by decoy ODN treatment. These results suggested that inhibition of the VEGF/VEGFR2 system in endothelial cells, which subsequently downregulates angiogenesis, is involved in the NF- κ B decoy ODN-induced reduction of periapical lesion development.

In conclusion, we found that inhibition of NF- κ B activity by decoy ODN treatment suppressed the development of experimentally induced periapical lesions with concomitant reduction of angiogenic responses in endothelial cells.

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Highlights

- Inhibition of NF- κ B activity with NF- κ B decoy ODN suppressed the development of experimental periapical lesions in rat molars.
- NF- κ B decoy ODN reduced the density of endothelial cells in the lesion.
- NF- κ B decoy ODN increased the Bax/Bcl-2 ratio and downregulated VEGF/VEGFR2 and chemokines in endothelial cells.
- Suppression of angiogenic responses is associated with the NF- κ B decoy ODN-induced lesion reduction.

Figure 1. Reduction of periapical lesion size by NF-κB decoy ODN treatment

(a-d) Representative radiographs and H-E stained photomicrographs in the NF-κB decoy ODN group (a, c) and NF-κB decoy scramble group (b, d). Scale bars, 200 μm. (e) Lesion size, as measured with digital radiomorphometry. * $P < 0.05$ ($n = 8$ each; Mann-Whitney U -test).

Figure 2. Reduction of CD31-immunoreactive area and NF-κB p65 activity by NF-κB decoy ODN treatment

(a, b) Representative photomicrographs showing CD31 immunoreactivity in the NF-κB decoy ODN group (a) and NF-κB decoy scramble group (b). Scale bars, 10 μm. (c) Percentages of immunostained area for CD31 in each group. $*P < 0.05$ ($n = 4$ each; Mann-Whitney U -test). (d) NF-κB p65 activity in lesions was measured with ELISA. $*P < 0.05$ ($n = 4$ each; Mann-Whitney U -test).

Figure 3. Immunohistochemical analysis of Bax and Bcl-2 in periapical lesions
(a-d) Representative photomicrographs showing Bax (a, b) and Bcl-2 (c, d) in the NF-κB decoy ODN group (a, c) and NF-κB decoy scramble group (b, d). Scale bars, 10 μm. **(e-g)**, Percentages of the immunostained area for Bcl-2 (e), Bax (f), and the Bax/Bcl-2 ratio in the immunostained area (g) of each group. * $P < 0.05$ ($n = 4$ each; Mann-Whitney U -test).

Figure 4. VEGF protein expression and *Bax*, *Bcl-2*, *CXCL1*, *CXCR2* and *VEGFR2* mRNA expression

(a) Total protein extracted from the periapical lesions was analyzed for VEGF expression by western blotting. The values represent arbitrary units calculated as band densities normalized to that of GAPDH and expressed as the ratio relative to the control. (b-g) Real-time PCR was used to quantify *Bcl-2* (b), *Bax* (c), *Bax/Bcl-2* ratio (d), *CXCL1* (e), *CXCR2* (f) and *VEGFR2* (g) mRNAs in CD31+ endothelial cells retrieved with LCM (n = 4, each group). Data were obtained by real-time PCR experiments and reflected the expression levels normalized to that of *GAPDH*.

**P* < 0.05 (Mann-Whitney *U*-test).