Title
Role of Fas Ligand in Apical Periodontitis upon Infection with Oral Anaerobes (Fusobacterium nucleatum)

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Role of Fas Ligand in Apical Periodontitis upon Infection with Oral Anaerobes

(Fusobacterium nucleatum)

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UCSF Division of Endodontics

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Yasaman Ravandoust
Role of Fas Ligand in Apical Periodontitis upon Infection with Oral Anaerobes

*Fusobacterium nucleatum*

By Yasaman Ravandoust, DDS

*Fusobacterium nucleatum* (*Fn*) is one of the most prevalent anaerobes found in endodontic infections. Our group has shown that *Fn* aggregates immune cells, upregulates the death-ligand FasL, and induces apoptosis (Jewett 2000, Fraser 2010, Huynh 2011). In this study, we investigated the role of FasL in regulating bone resorption upon murine pulp exposure and infection with *Fn* and *Prevotella intermedia* (*Pi*).

Mandibular first molar pulps from Wild-type, and FasL-deficient mice were exposed and infected with *Fn* and *Pi*. Mice with no pulp exposures were the control group. Animals were euthanized after 21 days. Hemi-mandibles were fixed in 10% phosphate-buffered-formalin, and subjected to micro-computed-tomography. The volumetric measurements of the periapical lesions on the mesial and distal roots were calculated and statistically analyzed with ANOVA and Post-hoc Tukey’s test.

Mesial and distal root periapical lesion sizes were significantly larger in Wild-type mice than in FasL deficient mice respectively (p<0.05).

a) Wild type mouse mesial root: 0.17±0.007;

b) FasL-deficient mouse mesial root: 0.12±0.007;
c) Wild type mouse distal root: 0.15±0.008;

d) FasL-deficient mouse distal root: 0.11±0.001

In conclusion, FasL plays an important role in the induction of periapical bone resorption induced by co-infection with *Fusobacterium nucleatum & Prevotella intermedia*. 
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INTRODUCTION

Apical periodontitis is primarily an inflammatory response to microbial progression from root canal to periradicular tissues. As a consequence of the encounter between bacteria and host defenses, inflammatory and immunologic changes take place and lead to development of apical periodontitis and bone resorption.

Most of the bacteria associated with primary endodontic infections and apical periodontitis are strict anaerobes. (1-2) *Fusobacterium nucleatum*, a gram-negative anaerobic rod, has been implicated in the pathogenesis of numerous infections throughout the body including that of root canal and periradicular tissue. Bergenholtz isolated the micro-organism from necrotic pulp in traumatized teeth. (3) Subsequently, *Fusobacterium nucleatum* was identified as the organism most frequently identified in root canals with necrotic pulp. (4-5) Sundqvist reported that *Fusobacterium nucleatum* was the single most prevalent isolated bacteria from root canals of teeth with periapical lesions. He also found a strong positive associations between *F. nucleatum* and *P. micros*, *P. endodontalis*, *Selenomonas sputigena*, and *Wolinella recta*. (2) *Fusobacterium nucleatum* is also known as a prominent pathogen in primary apical periodontitis, acute apical abscesses and post treatment endodontic disease. (6-10)

Chávez de Paz examined 28 cases seeking emergency treatment after initiation of root canal therapy. His report concluded that *F. nucleatum* appears to be associated with the development of the most severe forms of inter-appointment endodontic flare-ups. (11) Most recently, using pyrosequencing technology, Saber
demonstrated *Fusobacterium nucleatum* as the most abundant genera in symptomatic periradicular lesions. (12)

During the course of an infection, interrelationships develop between microbial species. Population shifts are often produced as a result of these interactions. These synergies are likely based on nutritional demands and may influence the pathogenicity of the polymicrobial root canal flora. (2,14) Positive interactions enhance the survival capacity of the interacting bacteria whereas negative interactions act as a negative feedback mechanisms that limit population densities.

One interesting synerigistic mechanism is called Coaggregation. Many microorganisms adhere directly to host surfaces, while other species adhere to bacteria already attached to the host surface. Coaggregation can favor colonization of host surfaces and facilitate metabolic interactions between partner microorganisms. (13)

*F. nucleatum* has demonstrated a wide array of coaggregation partners. For example, together with many Streptococci species, they can form a “corn on the cob” arrangement acting as a bridge between early and late oral colonizers. (16,17) Both autoaggregation (adherence to genetically identical cells) and coaggregation were observed in association with *F. nucleatum* isolated from acute endodontic infections. (13,15) Baumgartner *et al.,* (1992) showed that mixed culture of *F. nucleatum* with either *Porphyromonas gingivalis* or *Prevotella intermedia* was significantly more pathogenic than *F. nucleatum* in pure culture. (18)
Although the interaction between bacterial species and the host defense mechanisms is considered to be the key element in determining the status of health and disease in the mouth, neither the mechanisms of interaction among these bacteria (specifically in biofilms) nor their exact roles in pathogenesis of periradicular lesions are well understood. One possible theory for pathogenesis of *Fusobacterium nucleatum*, like many other Gram negative bacteria, is the presence of lipopolysaccharide (LPS). LPS is one of the first virulence factors to be identified in endodontic infections also known as endotoxin. (19) In 2006, Grenier showed that *F. nucleatum* lipopolysaccharide up-regulated the secretion of the pro-inflammatory cytokines interleukin-1beta, interleukin-6, and tumor necrosis factor-alpha. In addition, it increased phospholipase C and D activities, which likely contributed to the high levels of prostaglandin E2 detected in the cell culture supernatant. Lastly, the amount of matrix metalloproteinase-9 produced by macrophage-like cells was significantly increased by the lipopolysaccharide treatment. He concluded that this monocyctic responsiveness to lipopolysaccharide may be a key regulator of pathogenesis by this bacteria. (20)

Apoptotic immune cell death has also been recently reported as a potential mechanism for pathogenesis of different bacteria. Nalbant (2000) presented the data providing evidence for the induction of apoptosis among the majority of the T cells responding to *Aggregatibacter actinomycetemcomitans* (21) Several groups have reported on the immunosuppressive activities of *F. nucleatum*. (22-23) The
exact mechanism of immunosuppression is still unclear. It has been demonstrated that in addition to causing aggregation of peripheral blood mononuclear cells, *F. nucleatum* is able to induce apoptotic cell death in peripheral blood mononuclear and polymorphonuclear cells. (23)

Apoptosis, a form of active programmed cell death, is important in the development and regulation of the immune system, (24) characterized by a highly specific sequence of biochemical and morphologic changes including DNA fragmentation, decreased mitochondrial transmembrane potential, and formation of apoptotic bodies. Programmed cell death, as compared to necrosis, is associated with limited tissue inflammation, which allows the host to eliminate cells in an immunologically silent manner.

Two main pathways for the induction of apoptosis have been identified: the intrinsic and extrinsic pathways. Various stress factors, including oxidative stress and treatment with cytotoxic drugs, can activate the intrinsic pathway. The other pathway for the induction of apoptosis is the receptor-mediated or extrinsic pathway. Bacteria potentially act through this pathway. Two theories of the direct initiation of apoptotic mechanisms have been suggested: the *TNF-induced* (tumour necrosis factor) model and the *Fas-Fas ligand-mediated* model, both involving receptors of the *TNF receptor* (TNFR) family coupled to extrinsic signals. (25)
The Fas receptor (also known as Apo-1 or CD95) binds the Fas ligand (FasL), a transmembrane protein part of the TNF family. The interaction between Fas and FasL results in the formation of the death-inducing signaling complex (DISC), which contains the FADD, caspase-8 and caspase-10. In certain cell types (type I), processed caspase-8 directly activates other members of the caspase family, and triggers the execution of apoptosis of the cell. In other types of cells (type II), the Fas-DISC starts a feedback loop that spirals into increasing release of proapoptotic factors from mitochondria and the amplified activation of caspase-8 leading to activation of caspase 3 and ultimately apoptosis (Figure 1). (26)
Fig1: Apoptosis pathways (adopted from Edithors Peithos Nov 2007).
Jewett (2000) have shown that *F. nucleatum* can induce apoptotic cell death in peripheral blood mononuclear, polymorphonuclear cells (PBMCs), natural killer cell line, and Jurkat T cells. (23) A close relationship is observed between the ability of *F. nucleatum* to induce aggregation of the PBMCs and its ability to cause apoptotic cell death. Huynh and Jewett, in 2011, showed that aggregation is a critical step for the induction of death in PBMCs. Therefore, the bacteria might upregulate death ligand mediated destruction of PBMCs, and the aggregation might serve to bring the death ligands and their receptors into close proximity of each other for optimal death signaling in PBMCs.

This present study is based on the hypothesis that introduction of *F. nucleatum* to immune Jurkat cells lead to an increase of apoptosis through different death ligands including TNF alpha, FAS Ligand, and TNF-related apoptosis-inducing ligand.(Fig2).

Hence the purpose of this study was twofold:

1) to determine the role of death ligands in induction of apoptosis in Jurkat cells exposed to *F. nucleatum*

2) to evaluate the development of periapical lesions in Fas-L deficient mice infected with *F. nucleatum* compared to wild type mice.
Fig 2: Immune cell apoptosis as a result of aggregation through upregulation of death ligands.
MATERIALS AND METHODS

**Aim 1:** To determine the role of death ligands (TNFα, FasL, and TRAIL) in induction of apoptosis in Jurkat cells co-cultured with *F. nucleatum.*

**Cell lines, bacterial strains, antibodies, and reagents**

Jurkat T cells (an immortalized Leukemic T cell line) were maintained in RPMI 1640 supplemented with 1% sodium pyruvate, 1% nonessential amino acids, 1% penicillin-streptomycin and 10% fetal calf serum. *F. nucleatum* (PK 1594) obtained from Dr. P. Kolenbrander of the National Institutes of Health and grown in brain heart infusion broth containing 0.01% dithiothreitol in an atmosphere of 80% N₂, 10% CO₂ and 10% H₂ at 37°C. *F. nucleatum,* were treated with 1% paraformaldehyde for 1 hour at room temperature (formalin fixed). The bacteria were washed three times with 1× phosphate-buffered saline (PBS) (Invitrogen, Carlsbad, CA) to remove any remaining formaldehyde. (27)

Apoptosis activating FasL monoclonal antibodies and anti-FasL, anti-TRAIL, and anti-TNFα neutralizing monoclonal antibodies were purchased from RnD Systems (Minneapolis, MN). In co-culture experiments, Jurkat cells were co-cultured with formalized *F. nucleatum* at a ratio of 1:30.
**Apoptosis Assay**

Jurkat cells (2x10⁴) were cultured for 18 hours as follows: a) Jurkat cells alone, b) in the presence of apoptosis activating monoclonal antibodies (15 μg/ml), c) with *F. nucleatum*, and d) with *F. nucleatum* in the presence of anti-FasL, anti-TRAIL, and anti-TNFα neutralizing monoclonal antibodies (1 μg/ml). Cells were harvested and assessed for levels of apoptosis using the Caspase Glo 3/7 Kit (Promega, Madison WI) with a Multidetection Microplate Reader (Molecular Devices, Sunnyvale CA) following the manufacturer’s instructions.

**Aim 2:** To assess the role of FasL in *F. nucleatum* PK1594 induced apical periodontitis *in vivo*

**Animals**

Eight week old C57/BL6J male mice (wild type, n=6) and mice deficient for FasL gene (FasL -/-, n=3) were purchased from Jackson Laboratory (Bar Harbor, Maine) and maintained in UCSF Animal Facility (LARC). Animal protocols were reviewed and approved by the UCSF Institutional Animal Care and Use Committee. Average weight of mice was 18 grams.

**Periapical lesion induction**

For molar pulp exposure, mice were anesthetized with ketamine HCl (62.5 mg/kg) and xylazine (10 mg/kg) in sterile phosphate-buffered saline by intraperitoneal
injection. All lower left first molar pulps were exposed to the oral environment by using a #1/16 round bur under light and magnifying loop 3.5X. The pulp chamber was probed with a 10 endodontic file to confirm access of the bacteria to root canal system. Mice were divided into the following groups of 3 animals each:

Group A: FasL-deficient mice inoculated with a combination of *Prevotella intermedia* (ATCC25611) and *F. nucleatum* (PK1594);

Group B: Wild type mice inoculated with same combination of *P. intermedia* (ATCC25611) and *F.nucleatum* (PK1594).

Group C: Mice without pulp exposures served as controls. (This group included the contralateral molar tooth on each experimental animal, without any exposure)

*F.nucleatum* (PK1594) and *P.intermedia* (ATCC25611) were both grown on blood agar plates under anaerobic conditions (80% N₂, 10% H₂, and 10% CO₂), harvested, and cultured in Trypticase soy broth. The cells were centrifuged and resuspended in prereduced anaerobically sterilized Ringer solution. Bacteria were counted using a Neubauer hemocytometer (City, State). A total of 1 to 2 µL/tooth equal to 5x10⁷ of each bacteria were introduced into the molar using a micropipette. All animals were euthanized by CO₂ exposure and subsequent cervical dislocation on day 21 after pulp exposure. The mandibles were cleaned free of soft tissue, fixed in 10% phosphate-buffered formalin, stored in ethanol and subjected to microCT analysis (Xradia Inc., Pleasanton, CA).
Micro-CT Imaging

Hemi-mandibles were imaged using a Micro XCT™ (Xradia, Pleasanton, CA) at 4x magnification and at 75KVP. Computed tomography (CT) was used to study the 3D structure of the bone-PDL-root complex, and volume specifically representative of 0.5 mm from the root apices was evaluated. Virtual slices spaced 10 microns apart were assembled to reconstruct the 3D volume of the bone-PDL-tooth complex. Voxel size was approximately 10 × 10 × 10 µm, with a total scan time of 5 to 6.5 hours for each specimen.

Image Analysis

An algorithm to evaluate periapical lesion volume was developed using MATLAB (MATLAB version 7.10.0. Natick, MA: The MathWorks Inc., 2010). The bone-PDL-root complex representative of 0.5 mm from the apex of the mesial and distal roots were selected. The volumes of the respective roots were subtracted from the respective alveolar socket spaces. The resulting volumes were then predominantly equal to the volume of the periapical lesion in the mesial and distal roots. The algorithm is an active contour snake model, (28) in which a ballooning spline fit was used to evaluate the alveolar socket volume.
**Data Analysis:**

Results from both the caspase assay and microCT data, underwent one-way analysis of variance (ANOVA) (p<0.05), followed by the post-hoc Tukey’s test with the use of SPSS Statistical Editor Software (SPSS, Chicago, IL).

**RESULTS**

**Aim 1:** Diminution of caspase activity observed upon co-culturing Jurkats with *F. nucleatum* in the presence of neutralizing antibodies to TNFα, FasL, and TRAIL

The caspase levels (apoptotic marker) in different groups were as follows:

a) 183±4.5 (Jurkat cells alone),

b) 748±14.8 (Jurkat cells + FasL apoptosis activating monoclonal antibodies,

c) 432±10.2 (Jurkat cells + *F. nucleatum*, and

d) 316±13.3 (Jurkat cell+FasL+ *F. nucleatum*), 320±11.2 (Jurkat cell +TNFα + *F. nucleatum*) and 309± 14.1 (Jurkat cell +TRAIL + *F. nucleatum*).

There was a significant decrease in caspase activity upon the individual addition of FasL,TRAIL or TNF neutralizing monoclonal antibodies to the co-culture of Jurkats with *F. nucleatum* (*P<0.05). (Fig3)
Effect of neutralizing anti-FasL monoclonal Ab on Jurkat Apoptosis induced by *F. nucleatum*

![Bar chart](chart.png)

**Fig 3:** Abrogation of *F. nucleatum* induced Jurkat apoptosis when cultured in the presence of neutralizing monoclonal antibodies to FasL.
**Aim2:** Periapical periodontitis as a result of pulp exposure and infection with *F. nucleatum* and *P. intermedia* is significantly less in FasL-deficient mice compared to Wild type mice.

The role of FasL was further assessed in an *in vivo* pulp exposure model utilizing FasL deficient, and wild-type mice. Representative mid-sections of the molars obtained from micro-CT imaging in each group of mice showing the pulp exposure and periodontal ligament (PDL) space are shown in Figure 4. In the wild-type mouse section, periapical radiolucency can be seen in both roots of the first molar, whereas in the FasL deficient mouse section, there is only slight enlargement of the PDL space at the apex. (Figure 4)
Fig 4: MicroCT images of first molar after pulp exposure with *F. nucleatum* and *P. intermedia* in wild-type, FasL deficient and control mice.
The volumetric measurement (mm$^3$) of PDL widening as a representative of periapical periodontitis) were as follows:

a) Wild type mouse mesial root: 0.17±0.007;

b) FasL-deficient mouse mesial root: 0.12±0.007;

c) Wild type mouse distal root: 0.15±0.008;

d) FasL-deficient mouse distal root: 0.11±0.001

The differences in apical periodontitis between the mesial roots of the FasL-deficient and wild type mice were significant at $p<0.05$, as were the difference between distal root lesions. (Figure 5)
Fig 5: Volumetric analysis of the periapical lesion in the wild-type and FasL deficient mice.

DISCUSSION

*Fusobacterium nucleatum* is one of the most frequently isolated bacteria from root canals, and its pathogenicity has been shown to be synergistic with other oral anaerobes (2,4,8,10,14). Previous studies of *F. nucleatum* pathogenesis have suggested the immunosuppressive capability of this bacteria might be derived from its ability to induce cell death in immune cells, possibly by activating apoptotic pathways. (23) Bacterial induced cell death in human lymphocytes is a key
virulence factor which allows bacteria to fight the host immune system and establish pathogenesis. (29)

*F. nucleatum* ability to induce immune cell death appears to be mediated through immune cell aggregation. (27) Increased aggregation can aid in signaling for cell death by providing a bacterial scaffold that brings the cells in close proximity for cross receptor signaling between one cell and another. (27) In fact, our group, in unpublished data, had shown the upregulation of death ligands (TNFα, FasL, and TRAIL) that are potentially responsible for activation of apoptosis in immune cells.

In the present study, we aimed to show the role of these death ligands in signaling apoptosis both *in-vitro* and *in-vivo*. In our *in-vitro* experiments, we used Jurkat T cells as a representative of immune cells. Jurkats are an immortalized T cell line that have been previously utilized by other groups to study the Fas/FasL pathway in different systems, demonstrating inhibitory effects on apoptosis upon adding neutralizing FasL antibodies to the cell culture. (30)

Also in our *in-vitro* model, it appears that in conditions where the TNFα, FasL, and TRAIL were neutralized individually by their respective inhibitory monoclonal antibodies (d), induction of apoptosis by *F. nucleatum* was significantly reduced (P<0.05). However, there was not a complete abolishment of the caspase activity, when compared to background levels (a), suggesting that other mechanisms or combination of death ligands abolition may be involved in the apoptotic process.
Kaplan (2010) suggested outer membrane proteins Fap2 and RadD as potential inducer of immune cell apoptosis via *F. nucleatum*.(31)

Nalbant and Zadeh (2002) demonstrated that *A. actinomycetemcomitans* induces apoptosis of T lymphocytes by the Fas/FasL pathway. Similar to our findings, the authors showed that *A. actinomycetemcomitans* cell-free culture supernatant (CFCS) upregulated FasL on T cells. Blocking with anti-Fas monoclonal antibody led to significant decline, but not abolition of T cell apoptosis. The authors also suggest that other apoptotic pathways may be at play. (33)

Balto and Stashenko (2000) used the mouse model for *in-vivo* induction of periapical lesions.(32) We used only FasL deficient mice for our study since mice deficient in all three death ligands would be drastically immune-compromised and would not survive the 21 day waiting period intended to demonstrate lesion formation in our study. This time frame was also outlined by the Stashenko group as adequate time for development of apical periodontitis. (32)

Micro-computed tomography is an approach to non-invasively and non-destructively image and quantify bone in three dimensions. Balto demonstrated that micro-CT is a very precise technique allowing for the quantification of tooth/bone loss in the mice periapical area with accuracy equivalent to histological analysis. (32) Furthermore, it has been shown that three-dimensional volumetric quantification of apical periodontitis from the micro-CT images was highly correlated with two-dimensional cross-sectional measures of periapical areas. (34)

We opted to use micro-CT to compare the three-dimensional periapical bone/tooth
loss in both the mesial and distal first molar roots of wild-type and FasL-deficient mice after pulp exposure and infection with *F. nucleatum* and *P. intermedia*.

To simulate the clinical situations of endodontic infections with multiple communities of bacteria and their synergy for the *in-vivo* experiment, we chose to combine *F. nucleatum* and *P. intermedia*. (2) Our study, assessing the role of FasL in endodontic infections in an *in-vivo* pulp exposure model, showed that the size of the first molar periapical lesions were smaller in the FasL-deficient mice compared with wild-type mice after co-infection by *F. nucleatum* and *P. intermedia*. The *in vivo* results suggest that *F. nucleatum* is less pathogenic in an environment where FasL is not present. Perhaps, as demonstrated in the *in vitro* findings, *F. nucleatum* requires FasL to suppress the immune response in our mouse model. It is important to acknowledge that the findings of this study do not directly present any evidence that *F. nucleatum* is suppressing the immune response through the FasL pathway, and future immunohistologic studies are recommended to further investigate the molecular, immune/inflammatory response in these periapical lesions.
CONCLUSIONS

Our in-vitro study suggests the role, death ligands play in promoting apoptosis in immune cells after aggregation caused by Fusobacterium nucleatum. Our in-vivo experiment results demonstrate that wild-type mice with pulp exposures and co-infection by F. nucleatum and P. intermedia had significantly greater apical periodontitis compared with lesions in Fas-ligand-deficient mice.
REFERENCES


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