Dental Research and Management

Associations between HBD3 and Pro-Inflammatory Cytokines in Asymptomatic Irreversible Pulpitis

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Abstract

Objective: The aim of this study was to investigate the levels of Human Beta Defensin (hBD) 2 and 3, chemokine and cytokine expressions between teeth endodontically diagnosed with symptomatic irreversible pulpitis (SIP), asymptomatic irreversible pulpitis (ASIP) and normal pulps. We hypothesized that there would be a correlation between hBD's and the immunoregulatory response.

Design: Pulpal samples were collected with paper points. Six samples were obtained from normal teeth, 21 from SIP, 18 from ASIP. Levels of cytokines and beta-defensins were measured by Luminex technology and ELISA, respectively. Data were statistically analyzed using Kruskal-Wallis, Wilcoxon Mann-Whitney test and Spearman correlation test. Differences were considered significant at p<0.05.

Results: hBD-2 levels correlated with samples obtained from patients in the ASIP group, but not in the samples obtained from patients with SIP or the control group. HBD-3 concentrations associated with all of the cytokines and chemokines in both SIP and ASIP groups. However, in the normal group, hBD-3 correlated with only TNFα, IL-8, MCP-1, IL-1β, MIP-1a, RANTES, IL-17 in normal group. When comparing control levels of hBD-2 and hBD-3 with patients samples from either the ASIP or the SIP groups, hBD-2 and hBD-3 concentrations were highest in the ASIP group.

Conclusions: The hBD-2 and-3 were highly associated with the levels of the chemokines and cytokines in ASIP group. HBD-3 concentrations correlate with the levels of the chemokines and the cytokines in the SIP and ASIP groups.

Keywords: Human Beta Defensins; Pulp; Endodontics; Chemokines; Cytokines; Innate Immunity; Adaptive Immunity

Introduction

Dental pulp tissue is capable of innate and adaptive immune responses caused by various immunological conditions [1-3]. One host-defense system, involving the innate immune response upon exposure to the external environment, is the production of defensins [4]. Human beta-defensins (hBD) are small cationic antimicrobial peptides produced by epithelial cells and expressed by all human mucosa [5] including oral mucosa [6], odontoblasts [7] and pulp cells [8].

The mechanisms of the host immune defense against infections in human dental pulp (HDP) cells are not completely understood and the role that hBD play in protection of these cells has yet to be thoroughly explored.

Human beta-defensins have demonstrated immunologic response against gram-positive and -negative bacteria, mycobacteria, fungi, and certain enveloped viruses at low micromolar concentrations [9,10]. Human beta-defensins have antiretroviral activity by inhibiting HIV-1 infectivity of immunocompetent cells [11]. Additionally, hBDs can enhance adaptive immunity by acting as adjuvants and chemoattracting T cells, immature dendritic cells [5], neutrophils [12] and macrophages [13]. Human beta-defensins-2 is mediated through nuclear factor kappa-light-chain-enhancer of activated B cells (NF-KB) and mitogen-activated-protein-kinases (MAPK) pathways [14,15], while hBD-3 is dependent upon epidermal growth factor receptor (EGFR) activation [16,17].

There are many studies in the medical literature that have linked hBD-2 and hBD-3 with cytokine and chemokine production [18-21].

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Human beta-defensins-2 are highly expressed when the human dental pulp cells are stimulated with IL-1β or TNFα [22]. Dommisch et al. 2007 [23] reported that hBD-2 stimulation of odontoblasts and dental pulp stem cells led to up-regulation of the IL-6 and IL-8 mRNA.

Most of the above mentioned studies were in-vitro investigations. To date only one clinical dental study investigated the association between inflamed pulp and hBD's in HDPs [8]. Since bacteria from carious lesions elicit inflammatory and immunological responses in the dental pulp [24], the current authors reasoned that the relative concentrations of hBDs and inflammatory cytokines might modulate the outcome of pulp pathosis. A better understanding of pulpal immune response at different stages of inflammation may allow development of an immune system-based pulp therapy in the future. To begin testing this hypothesis, the current authors examined hBDs and cytokine profiles of symptomatic and asymptomatic irreversible pulpitis in human teeth.

To the best of our knowledge, no previously published work has examined hBDs or cytokine and chemokine profiles involved in endodontic pulpal pathosis. Thus, the aim of this study was to investigate the levels of hBD-2 and hBD-3, and chemokine and cytokine expression levels in pulps from teeth endodontically diagnosed with symptomatic irreversible pulpitis, asymptomatic irreversible pulpitis or normal pulps. We hypothesized that there would be a correlation between hBD's and the immunoregulatory response in the pulp.

Materials and Methods

Patient selection

this study was approved by the Institutional Review Board (IRB), Case Western Reserve University, Cleveland, Ohio, and written informed consent was granted from all patients. Patients undergoing non-surgical root canal treatment from August 2013 to April 2014 were selected. The investigation did not alter the treatment plan of any patient. Patients were provided with information about the purpose of the study and written informed consent was obtained. Participants had to have met the following criteria: American Society of Anesthesiologists (ASA) physical status 1 or 2, no history of known allergies, not pregnant, non-smokers, age between eighteen and sixty five, healthy periodontal status and restorable teeth. Exclusion criteria: younger than eighteen years or older than sixty five, patient on antibiotics, patients with any known allergies, pregnancy, diabetes, immunocompromised patients, any periodontal probing depth greater than 5mm or teeth with a furcation or trifurcation involvement, teeth diagnosed with a necrotic pulp, previously initiated endodontic treatment and/or previously endodontically treated teeth.

Each patient's pulpal and periradicular status were evaluated by cold test (HYGIENIC®; ENDO-ICE®; Coltène/Whaledent Inc., Cuyahoga Falls, OH, USA) and electric pulp test (EPT) (Vitality Scanner; SybronEndo, Orange, CA, USA) to assess pulp vitality. Percussion, palpation, and periodontal examinations were performed. Digital periapical and bitewing radiographs of the tooth in question (Planmeca® ProSensor™; PLANMECA USA, Inc., Roselle, IL, USA) were obtained.

The following type patients were subsequently included: patients diagnosed with symptomatic irreversible pulpitis (SIP) or asymptomatic irreversible (ASIP) pulpitis where excavation of caries resulted in pulpal exposure. The definitions of the SIP and ASIP were described in a previous study [25]. No traumatized teeth were included in this research. As a negative control, samples were also taken from six teeth which had no clinical or radiographic evidence of pulpal and periapical pathosis but needed routine endodontic treatment for prosthodontic reasons. Briefly, the clinical characteristics of the cases included subjective and objective findings which are described:

SIP: included sharp pain upon thermal stimulus, lingeringpain (often 30 seconds or longer after stimulus removal).

ASIP: these cases had no clinical symptoms and usually respond normally to thermal testing deep caries resulted in exposure following removal [26].

Control: normal pulp, where the teeth were symptom-free, healthy and free of caries.

Operative procedure & site selection

the sampling procedure is a modified procedure as described by Martinho et al. 2008, 2015 [27,28]. In brief, teeth were mechanically cleaned and disinfected by 0.12% chlorhexidine (Peridex™; 3M™, USA). After local anesthesia and rubber dam placement, an access opening was made using a sterile size #2 round carbide bur (Dentsply Maillefer, Tulsa, OK, USA) in a high speed hand piece to expose the pulp. An ENDO-Z bur (Dentsply Maillefer, Tulsa, OK) was used to deroof the pulp chamber. Paper points size 35/0.02 taper (Lexicon ®; DENTSPLY Tulsa Dental Specialties, John City, TN, USA) were introduced into the pulp chamber and left for 60 seconds. The procedure was repeated with 4 paper points. The paper points were placed into Eppendorf tube (Eppendorf Tubes®, Lakewood, OH, USA) containing 400 μL of phosphate-buffered saline (PBS) (Gibco® PBS pH7.4,Grand Island, NY, USA) centrifuged at 10000 g at 4°C for 15 minutes and stored at -70°C until use.

Bicinchoninic acid assay (BCA assay)

Bicinchoninic acid assay was first introduced by Smith et al. [29] and is a sensitive methodology for protein quantification [30].

Total proteins in the samples were measured using the BCA protein assay kit (Pierce, Rockford, IL, USA) following the manufacturer's instructions.

Enzyme-link immunosorbent assay (ELISA)

Levels of hBD-2 and hBD-3 were measured by sandwich enzyme-linked immunosorbent assay (ELISA). Ninety-six-well immunoplates (R&D, Minneapolis, MN) were coated with 100 μL goat anti–hBD-2 or rabbit anti–hBD-3 antibodies (Peprotech, NJ) diluted to 1 mg/L in 0.05 mol/L carbonate buffer, pH 9.6, 4°C, for 18 h. Subsequently, the sample were blocked with 200 μL 1% bovine serum albumin in PBS at room temperature [about

20-25°C] for 10 minutes. After washing three times with 200 μL phosphate buffered saline (PBS), 0.01% Tween 20, 50 μL of test samples + 50 μL of PBS per well were added and incubated at room temperature for 60 min. Plates were washed three times with PBS, 0.01% Tween 20 and wells incubated at room temperature with 100 μL biotinylated goat anti-human BD-2 or biotinylated rabbit anti-human BD-3 (Peprotech, NJ) diluted to 0.2 mg/L in PBS, 0.01% Tween 20 for 30 min. Plates were washed three times with PBS, 0.01% Tween 20 and 100 μL/well streptavidin HRP (R&D, Minneapolis, MN) was added. Plates were then incubated at room temperature for an additional 30 min, washed three times as described above, and incubated with 100 μL of Reagent (A+B) (R&D, Minneapolis) in the dark at room temperature for about 15 min. Reactions were stopped by adding 50μL of stop solution (R&D, Minneapolis). Absorbance was measured at 450nm in a microplate reader (Bio-Rad Model 680). Human beta defensins were quantified by simultaneous ELISA using recombinant hBDs as calibrators.

Cytokine and Chemokine measurement by Luminex

the Clinical Translational Science Collaborative Bioanalyte Core utilizes software that allows for the standardization between samples and between studies longitudinally; all values are evaluated using a standard curve which has been validated as a comparison of multiple assays to assure consistency between analyses (found at: http://casemed.case.edu/ctsc/cores/bioanalyte.cfm). The standard curve for sample cytokine and chemokine concentration determination was used on the basis of the standard curve using Bio-Plex Manager 6.1 (Bio-Rad Laboratories, Hercules, CA).

Luminex analysis (Luminex®100 platform; Austin, TX, USA) of the samples was performed using the Cytokine and Chemokine Human 10-plex panel multiplex assay (Novex®, Life technologies™, Grand Island, NY, USA). These included: tumor necrosis factor- α (TNFα), interleukins (IL-1β, IL-6, IL-8, IL-10, IL-17, IL-17F), monocyte chemotactic protein-1 (MCP-1; also known as chemokine (C-C motif) ligand 2 CCL2), macrophage inflammatory protein (MIP-1a, also known as CCL3) and regulated on activation normal T cell expressed and secreted (RANTES, also known as CCL5). Levels of the cytokines were normalized with total protein as measured by BCA assay and expressed as pg/mg of total proteins.

Normalization Methods

Total protein

To normalize the data the levels of the hBDs, cytokines and chemokines were expressed as per mg of total proteins The total concentration of protein was measured in each specimen using a bicinchoninic acid (BCA) assay as per manufacturer's protocol (Pierce, Rockford IL). This assay has a reported dynamic range of 20–20,000 μg/ml and has a 14.7% mean coefficient of variance for repeat testing across 14 different human and non-human purified protein targets. Specimens were diluted 1:10 and 1:100 in PBS and run in duplicate. Colorimetric detection of test specimens was normalized to background specimens that contain extraction buffer only. Total protein concentration was estimated using an 8-point standard curve and is expressed as μg/ml. The ratio of immune marker concentration to total protein concentration was then calculated and expressed as [pg of immune marker]/[mg of total protein].

Sample calculation

sample size calculation was performed before the beginning of the study using the SAS Power and Sample Size 3.1 of SAS (Statistical Analysis System) software for Windows version 9.1.3 (SAS Institute Inc. Cary, NC, USA). Expecting the minimum correlation of 0.60 with power of 0.80 and alpha of 0.05 and one-sided test, the minimum sample size for the experimental cases was 15 root canals.

Statistical analysis

demographic characteristics were expressed as the means and standard deviations and Chi-square test. Results of hBDs, chemokines and cytokines were normalized by the equation: (cytokine or chemokine or HBD expressed) / total protein (BCA assay). Data were statistically analyzed using Kruskal-Wallis, Wilcoxon Mann-Whitney test and Spearman correlation test. The level of statistical significance was set at 95% confidence interval ($p < 0.05$), and the statistical analysis was calculated using Prism 6.0 software (GraphPad Prism version 6.0 for Windows, San Diego, CA, USA).

Results

Table 1 details the demographic distribution and the mean age. To determine whether the variables were statistically independent we performed Pearson Chi Square test and the result found not to be significant ($\chi^2 = 1.534$, p=0.464).

Table 2 details the mean, median and standard deviation of levels of hBD's, cytokines and chemokines in normal, SIP and ASIP groups.

Table 3 details the analysis between levels of normalized hBD-2 in comparison to chemokines, cytokines with statistical analysis. To normalize the data the levels of the hBDs, cytokines and chemokines were expressed as per mg of total proteins. There was no correlation between the levels of hBD-2 in comparison to the cytokines and chemokines in the normal and SIP groups; however, in the ASIP group there was a correlation between the levels of hBD-2 and TNFα, IL-6, IL-8, IL-10, MCP-1, IL-1β, MIP-1a, RANTES, IL-17 and IL-17F.

Table 4 details the levels of normalized hBD-3 in comparison to chemokines, cytokines with statistical analysis. In the SIP and ASIP groups there was a correlation with TNFα, IL-6, IL-8, IL-10, MCP-1, IL-1β, MIP-1a, RANTES, IL-17 and IL-17F (all the cytokines, chemokines studied). For all groups, there was a correlation with the levels of hBD-3 to TNFα, IL-8, MCP-1, IL-1β, MIP-1a, RANTES and IL-17.

Discussion

To the best of our knowledge, this is the first endodontic clinical study that investigated the role of hBDs in relation to pulpal

Demographic Characteristics			
	Medically Healthy Subjects		
Pulpal Diagnosis	**Normal Pulp**	**Symptomatic Irreverisble Pulpitis**	**Asymptomatic Irreversible Pulpitis**
Number of patients	6	21	18
Age (Years): Mean± Standard Deviation	38±19	35±15	40±20
Gender			
Male	2	7	3
Female	4	14	15

Chi Square test (X²) = 1.534, 2; P-Value= 0.4644

Table 1: Demographic characteristics and endodontic diagnosis of the healthy patients.

	Normal Pulp			**Symptomatic Irreversible Pulpitis (SIP)**			**Asymptomatic Irreversible Pulpitis (ASIP)**		
	Mean	Median	SD	Mean	Median	SD	Mean	Median	SD
HBD2	0.17	0.188	0.095	0.707	0.359	1.065	1.186	0.580	1.549
HBD3	3.22	2.258	2.99	3.666	1.331	5.319	8.712	3.172	18.16
TNFα	0.11	0.098	0.073	0.162	0.064	0.197	0.194	0.089	0.2239
IL-1β	0.12	0.099	0.081	0.167	0.078	0.165	0.229	0.096	0.2632
IL-6	0.092	0.079	0.058	0.136	0.059	0.140	0.160	0.079	0.185
IL-8	0.122	0.107	0.074	0.175	0.073	0.191	0.219	0.097	0.2518
IL-10	0.090	0.066	0.059	0.111	0.0513	0.113	0.141	0.078	0.16
IL-17	0.093	0.079	0.056	0.136	0.066	0.151	0.173	0.085	0.1516
IL-17F	0.116	0.085	0.077	0.156	0.074	0.174	0.212	0.101	0.242
MIP-1a	0.919	0.069	0.071	0.126	0.056	0.146	0.156	0.082	0.1729
MCP-1	0.120	0.095	0.068	0.171	0.076	0.181	0.220	0.099	0.232
RANTES	0.101	0.086	0.055	0.164	0.070	0.197	0.178	0.088	0.1972

Table 2: Mean, median and standard deviation (SD) for Human Beta Defensin (HBD), Cytokines and Chemokines Levels (pg/mg) with various pulpal diagnoses.

	hBD-2 in Normal Pulp		**hBD-2 in SIP**		**hBD-2 in ASIP**	
	Correlation Coefficient	**P-Value**	**Correlation Coefficient**	**P-Value**	**Correlation Coefficient**	**P-Value**
TNF a	0.08571429	0.919444	0.3467532	0.123571	0.7151703	<0.0001*
IL-6	0.02857143	1	0.3363636	0.135997	0.748194	<0.0001*
IL-8	0.1428571	0.802778	0.2662338	0.243399	0.7172343	<0.0001*
IL-10	-0.08571429	0.919444	0.3311688	0.142531	0.8039216	<0.0001*
MCP-1	0.08571429	0.919444	0.3571429	0.111981	0.7853457	<0.0001*
IL-1b	0.08571429	0.919444	0.2727273	0.231656	0.74613	<0.0001*
MIP-1a	0.02857143	1	0.3545454	0.114802	0.7956656	<0.0001*
RANTES	0.08571429	0.919444	0.3103896	0.170877	0.8121775	<0.0001*
IL-17	0.02857143	1	0.2532468	0.268018	0.6759546	0.002074*
IL-17F	0.02857143	1	0.3662338	0.102504	0.7791538	<0.0001*

Statistical significance: *P-value <0.05

Table 3: Correlation of Human Beta Defensin-2 with cytokines and chemokines by Spearman's rank correlation coefficient.

cytokines and chemokines. Since there have been many medical studies which have linked hBD-2 and hBD-3 with cytokine and chemokine production suggesting these hBDs link innate and adaptive immunity [5,31,32], we focused on correlating hBD levels with the cytokine/chemokine panel used in this current investigation. Results supported the hypothesis that there is a correlation between hBD's and the immunoregulatory response.

At this stage, it is not clear why the levels of hBD-2 correlated only in asymptomatic, but not in symptomatic, irreversible pulpitis compared to the normal pulp (Table 3). In contrast, the levels of hBD-3 correlated in both ASIP and SIP (Table 4). These observations imply that there are differences between SIP and ASIP and the involvement of hBD's and cytokines, chemokines. Studies are on-going in our laboratory to explore why SIP and ASIP may be different and the potential contribution of alternative inflammatory mediators such as neuropeptides and microbial differences between the two cases (SIP and ASIP groups).

The results of this study agree with Dommisch et al. who reported, in their in-vitro study, that hBD-2 stimulated the gene expression of pro-inflammatory cytokines [23]. Our findings also agree with Kim et al. [22] who reported that there was a correlation between TNF-α and hBD-2. The reason why, in the SIP group there was a correlation between the hBD-3 and cytokines, chemokines (Table 4) but not hBD-2 levels with the cytokines and the chemokines, needs further investigation (Table 3). In both SIP and ASIP groups (Table 4) there was a significant correlation between hBD-3 and all the chemokine and cytokines. Since the levels of hBD-3 were correlated in SIP and ASIP groups, this again might suggest that perhaps hBD-3 may play an even more extensive role in immunoregulation than previously reported in the endodontic literature.

There are many speculations and possibilities as to why an inflamed pulp might be symptomatic or asymptomatic. These possibilities include the presence of endotoxins [33], the immunoregulary response [34-36], and pathogens of various microbial progression [24,37-39]. The latter describes how various microbes elicit different immunologic responses. In the periodontal literature, hBD-3 was reported to bind to a strain of Porphyromonas gingivalis and attenuated a proinflammatory response [40]. The correlation between hBD-3 and the microbe was significantly higher than hBD1 or hBD-2 resulting in significant attenuation of the interleukin (IL)-6, IL-10, granulocyte macrophage colony stimulating factor (GM-CSF) and tumor-necrosis factor-a (TNF-α).

Other similar studies [14,40 41] suggest that different microbial pathogens elicit different immunological responses. In future studies, differences between microbial pathogens in symptomatic and asymptomatic irreversible pulpitis should be better explored.

	hBD-3 in Normal Pulp		**hBD-3 in SIP**		**hBD-3 in ASIP**	
	Correlation Coefficient	**P-Value**	**Correlation Coefficient**	**P-Value**	**Correlation Coefficient**	**P-Value**
TNF a	0.8857143	0.033333*	0.8168831	<0.0001*	0.7605779	0.000248*
IL-6	0.7714286	0.102778	0.8753247	<0.0001*	0.750258	0.000335*
IL-8	0.9428571	0.016667*	0.8090909	<0.0001*	0.7750258	0.000158*
IL-10	0.7142857	0.136111	0.8363636	<0.0001*	0.6965944	0.001318*
MCP-1	0.8857143	0.033333*	0.8584415	<0.0001*	0.750258	0.000335*
IL-1b	0.8857143	0.033333*	0.9012987	<0.0001*	0.750258	0.000335*
MIP-1a	0.9428571	0.016667*	0.8285714	<0.0001*	0.7420021	0.000423*
RANTES	0.8857143	0.033333*	0.8090909	<0.0001*	0.7626419	0.000233*
IL-17	0.9428571	0.016667*	0.8584415	<0.0001*	0.8575851	<0.0001*
IL-17F	0.7714286	0.102778	0.8909091	<0.0001*	0.7894737	<0.0001*

Statistical significance: *P-value <0.05

Table 4: Correlation of Human Beta Defensin-3 with cytokines and chemokines by Spearman's rank correlation coefficient.

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We do agree that the sample size for our normal group was smaller compared to the other two groups (SIP and ASIP). We would have preferred to have had a larger sample size for normal subjects, but it is not easy to get samples from teeth with normal pulps. However, the previous studies [8,42,43] had a similar sample size as ours for their control group and based on these previous studies we performed our power calculation of the study as detailed in the Methodology Section.

We used paper points for our clinical sampling technique. Previous clinical studies (excluding extraction of teeth) have used paper points [27,28,44-50], cotton pellets [34,51], barbed broach [43] to measure cytokines, chemokines, neuropeptides and exotoxins in the root canal system. Currently there are no "gold standard techniques" for sampling pulpal tissue, and to date, the current molecular-based methods are still under continuous improvement [52].

Future studies evaluating microbial differences in root canals and the concomitant host responses could provide an interesting contribution to the understanding of the host-pathogen relationship.

Conclusions

Human beta defensin-2 and hBD-3 were associated with the cytokines and chemokines in ASIP group. HBD-3 concentrations correlated with the levels of the chemokine and the cytokines in the SIP and ASIP groups.

Acknowledgement

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