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Expression of NOD1 Protein in Odontoblast Cells upon Stimulation with Lipoteichoic Acid

Research Article

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Abstract

Introduction: Pathogen recognition receptors are able to identify microbes and their products and play a role in innate immunity. NOD1 has been shown to be present in the odontoblast cells. The present study was conducted with the aim to study the change in expression of NOD1 in odontoblast cells upon stimulation with lipoteichoic acid.
Materials and Method: The KN3 cells line was used for the analysis. The study consisted of three groups, the unstimulated cells, KN3 stimulation with 1µg/ml of lipoteichoic acid and KN3 stimulation with 10µg/ml of lipoteichoic acid for 2 hours. Qualitative analysis was done by observing the cells under a phase contrast microscope and quantitative analysis was done by the ELISA test.
Results: It was observed that stimulation with LTA increased the expression of NOD1 in odontoblast cells and this expression of NOD1 increased with the increase in the concentration of LTA. The highest expression of NOD1 was observed in the cells that were stimulated with 10µg/ml of LTA for 2 hours (440.8 +/- 66.274).
Conclusion: NOD1 expressed in odontoblast cells is capable of recognising lipoteichoic acid. There is a concentration dependent upregulation of NOD1 in odontoblast cells that are stimulated by lipoteichoic acid.

Keywords: Lipoteichoic Acid; NOD; Odontoblast; Pattern Recognising Receptors; Pulpitis.

Introduction

Tissue damage caused by various factors such as microbial infection, burns, infractions induces a protective inflammatory response in an attempt to remove the stimuli and repair the damaged tissue. A proper activation of both innate and adaptive immunity will generate a protective response in the body against pathogens. Charles Janeway Jr. in 1989 proposed that there exist receptors that are capable of detecting products of microbial origin. These receptors are located on the innate immune cells such as dendritic cells, macrophages, monocytes, neutrophils and epithelial cells [1]. These are the so-called Pattern Recognising Receptors (PRR) that are proteins capable of recognising molecules that are frequently associated with pathogens; called the Pathogen Associated Molecular Pattern (PAMP) [2]. These receptors can be found associated with the cell membrane, the endosomal membrane, the cytosol, as well as extracellularly in secreted forms [3]. There are four major subgroups of PRRs; Toll-like receptors (TLRs), Nucleotide-binding oligomerization domain (NOD), retinoic acid-inducible gene 1 (RIG-1) -like receptors and C-type lectin receptors (CLRs)[4].

NOD1 and NOD2 belong to the Nod-like receptor (NLR) family and are present intracellularly. In vitro studies using cellular models, murine in vivo models as well as genetic susceptibility studies done on humans have demonstrated the importance of NOD1 and NOD2 in generating a host defense against pathogens [5].

The principal driving force for a pulpal response is the system's immune reaction to the microbes that invade the pulp which enhances the production of inflammatory mediators [6]. It has been suggested that the PRRs are able to identify the molecular patterns on these invading microorganisms. TLR2, TLR4, NOD2 and NOD1 have been detected in human dental pulp fibroblasts.

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[7]. It has been proposed that NOD1 is responsible for the upregulation of chemokine expression via p38 ap 1 signalling pathway which may play a role in initiation and progression of pulpitis [8]. Further, NOD1 AND NOD2 are also functionally expressed in human periodontal ligament cells and can trigger innate immune response [9]. However there has been no study that has evaluated the expression of NOD1 in odontoblast cells upon interaction with lipoteichoic acid (LTA) which is found in the cell wall of gram positive bacteria.

Previously our team has a rich experience in working on various research projects across multiple disciplines [10-24] Now the growing trend in this area motivated us to pursue this project.

Hence, the present study was conducted with the aim to study the expression of NOD1 in odontoblast cells upon stimulation with lipoteichoic acid.

Materials And Method

Cell Culture

The odontoblast cells KN3 were obtained from ATCC. The culture media and other microbial broth was purchased from Himedia Laboratories. KN3 cells were cultured in normal medium comprising of Eagle's minimal essential medium(α-MEM) with 10% FBS, 500 U/mL penicillin, 500µg/mL streptomycin, and 25µg/mL amphotericin B at 37°C in a humidified atmosphere of 5% CO_2. Approximately 8×10^4 cells/ml in medium were seeded in wells of 24 well tissue culture plates and incubated until confluent monolayers developed.

Odontoblast Cell Stimulation

There were three groups in the study

Group A: Stimulation of cells with 1µg/ml of LTA for 2 hours
Group B: Stimulation of cells with 10µg/ml of LTA for 2 hours
Group C: Unstimulated cells.
Lipoteichoic acid was purchased from Sigma Aldrich Company. After 4 weeks of culturing
process, cultures were stimulated for 2 h with 1µg/ml and 10 µg/ml LTA. The cell images were viewed in phase contrast microscope.

Estimation of NOD 1

The NOD 1 expression was estimated using the manufacturer's instruction kit (CUSABIO Company). For the quantitative determination of NOD1 concentrations in cell lysates, the media was removed from the cells and rinsed with ice-cold PBS (pH7.2-7.4). The cells were scrapped off the plate and transferred to an appropriate tube. The cell suspension was diluted with 1xPBS (pH7.4) until cell concentration reached 100 million/ml. It was then stored overnight at -20°C. After two freeze-thaw cycles to break up the cell membranes, the cell lysates were centrifuged for 5 minutes at 5000xg, 2 -8°C. The supernatant was used for the estimation of NOD1 protein. 100µl of standard/sample per well was added and incubated for 2 hours at 37°C. The liquid was removed and added 100µl of Biotinantibody was added to each well and incubated for 1hour at 37°C. The plates were washed three times with washing buffer and 100µl of HRP-avidin(1x) was added to each well and incubated for 1 hour at 37°C. This was followed by addition of 90µl of Tetramethylbenzidine (TMB) Substrate to each well and incubation for 15-30 minutes at 37°C. 50µl of Stop Solution was added to each well and the optical density of each well was measured within 5 minutes using a microplate reader (MINDRAY MODEL) set to 450 nm.

Statistical Analysis

For statistical analysis of data, multiple comparisons were performed using one-way analysis of variance (ANOVA) followed by the Fisher's Least Significant Difference test for post hoc analysis. Statistical significance was accepted at a level of $P<0.05$. Data was analyzed using SPSS (version 22.0).

Results

It was observed that stimulation with LTA increased the expression of NOD1 in odontoblast cells and this expression of NOD1 increased with the increase in the concentration of LTA (Table 1, Fig 1) The highest expression of NOD1 was observed in the cells that were stimulated with 10µg/ml of LTA for 2 hours (440.8 +/- 66.274). These results were statistically significant.

Discussion

Our institution is passionate about high quality evidence based research and has excelled in various fields [14, 25-34].

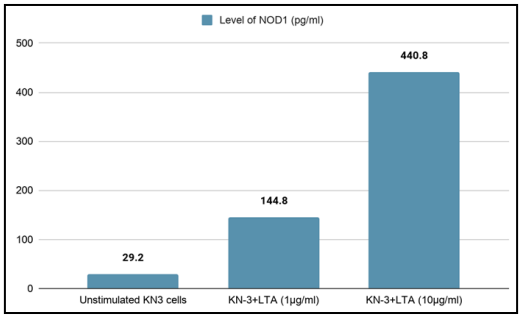
In response to caries related bacteria and their components that cause pulpitis, there is an increase in the expression of pro inflammatory mediators [35]. It has been previously reported that in the early phase of host innate immune response, the TLRs recognise PAMPs which elucidates an expression of inflammatory genes. Studies have suggested the expression of TLR2 and TLR4 in the cells of the pulp tissue, especially odontoblasts and fibroblasts [36, 37]. Here we have demonstrated an upregulation in the expression of NOD1 in odontoblast cells upon stimulation with LTA suggesting its influence in the generation of pulpal response. Bacterial invasion accompanying the advance of dental caries sequentially initiates immune reactions to develop pulpitis.

The most frequently isolated organism from a deep carious lesion is gram positive rods of which lactobacilli constituted 91.9% of

Table 1. Level of NOD1 in cell lysate expressed as mean and standard deviation. P value was 0.02.

Groups	N	Mean (pg/ml)	Standard deviation
Unstimulated KN3 cells	3	29.2	14.306
KN3+LTA (1µg/ml)	3	144.8	10.513
KN3+LTA (10µg/ml)	3	440.8	66.274

Figure 1. Graphical representation of NOD1 levels in cell lysate. The X axis represents the level of NOD1 and the Y axis represents the three groups. Statistical analysis shows p<0.01 depicting that the results are statistically significant.



the total micro flora at the pulpal site [38]. LTA is a component of the cell wall of gram positive bacteria [39]. It is an amphiphilic molecule consisting of a poly glycerol phosphate with a complex glycolipid group attached and is anchored to the cell membrane of gram positive bacteria by hydrophobic forces. Lipopolysaccharide and Lipoteichoic acid bind to CD14 and activate signalling by TLRs [40]. It has been demonstrated that lactobacillus LTA induced TNF production by TLR2 [41].

Hence in this study model LTA stimulation was done. Further, there has been no study to demonstrate the expression of NOD1 in response to LTA making this a novel approach.

NOD1 and NOD2 are present intracellularly and act as sensors for bacterial peptidoglycans. Studies have shown that NOD1 mainly recognises g-D-glutamyl-meso-diaminopimelic acid found predominantly in Gram-negative bacteria and NOD2 detects muramyl dipeptide. This study has shown the ability of NOD1 to be expressed by LTA. A previous study has shown an increased expression of NOD2 in inflamed pulp and LTA stimulated odontoblast cells.It was observed that 10µg/ml LTA augmented NOD2 gene expression in a time-dependent manner and this expression significantly increased after 2 hours of stimulation [42]. Hence the two concentrations considered under this study model were 1µg/ml and 10µg/ml and the time duration for the stimulation was set at 2 hours. Primary odontoblasts have difficulty in passage culture because of replicative senescence and too little cells isolated from dental pulp hence the KN-3 cell line which is an established rat odontoblastic cell line was used.

Our study has shown that NOD1 expressed in odontoblastic cells recognises LTA present in gram positive bacteria. This might lead to production of several chemokines responsible for pulpal inflammatory response. Further, it has been previously demonstrated that the NOD1 expression in odontoblast cells is stronger than the NOD2 expression [8].

Till date NOD1 and NOD2 are among the best studied NLR family proteins The modulation of innate immune response targets is one of the major goals in the development of novel therapeutics for human autoimmune and chronic inflammatory disease In this context NOD1 and NOD2 are of particular interest since they recognize distinct bacterial structures and directly activate multiple inflammatory pathways. Theoretically, chemical antagonists off NOD1 and NOD2 could have applications in several acute and chronic disease in which dampening the pro inflammatory responses of the innate immune system might be beneficial.

Conclusion

NOD1 expressed in odontoblast cells is capable of recognising lipoteichoic acid. There is a concentration dependent upregulation of NOD1 in odontoblast cells that are stimulated by lipoteichoic acid.

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