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Research paper

Investigation of Intestinal Enzyme Activity and Effects of Non-starch Polysaccharide on it

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Abstract

Background and Purpose: Shigella is a human shigellosis and its lipopolysaccharide is identified by 4TLR. The 4TLR is a family of pseudo-TOLL receptors and many immune routes are triggered by stimulating these receptors. Many studies show increasing of 4TLR expression in Mesenchyme stem cells under the influence of lipopolysaccharide. The main objective of this study was to identify the appropriate lipopolysaccharide of Shigella strains by stimulating the immune system for vaccine studies. Materials and Methods: In this experimental study, the stem cell of human Mesenchymal derived from bone marrow was treated by three dilution of 0.1, 0.01, and 0.001 extract of Shigella strains (Flexneri, Dysentery and Sonnei) containing lipopolysaccharide. Then, the expression of 4TLR at RNA level was evaluated by RT-PCR and Q-PCR techniques. Cells treated with phosphate buffer saline were considered as control group. Findings: The expression of 4TLR was observed in all treatments groups except for treatment groups with relative concentration of 0.001 sonnei and dysentery as well as control group. Changes in 4TLR expression were dose-dependent on all treatment groups. The highest expression was related to the treatment with Shigella Flexneri extract and the smallest was related to Shigella sonnei. The use of pure lipopolysaccharide of Escherichia coli as a positive control showed that the lipopolysaccharide in Shigella extract is responsible for increasing the expression of 4TLR. Conclusion: given the increased expression of 4TLR by Shigella extract, this extract is recommended to increase the efficacy of the vaccine.

Keywords: *Cell Extracts, Laboratory Conditions, Intrinsic Immunity, Lipopolysaccharide, Shigella.*

1. Introduction

One of the largest families of bacteria is Enterobacteriaceae family, which due to their higher prevalence in humans and animals, extensive studies have been done on them. All members of this family have gram-negative bacillary and do not produce spores. The bacterial genital diversity is high in this family, from E. coli, some of which are natural intestinal flora, to Salmonella, Shigella, Klebsiella, Proteus and Enterobacter, which are considered to be pathogenic for human are variable (1, 2). Shigella bacteria, like other Enterobacteriaceae bacteria, have short and warm gram-negative bacillary that sometimes are seen as a chain beside each other. These bacteria lack flagellum, include capsule and are non-movable (2, 3). Invasive bacteria have the ability to penetrate into epithelial cells in vitro and vivo. Once the bacteria entered the host, several reactions begin, on which the process of the disease depend (4). There is a place within the epithelial space of intestine where Shigella encounters immune cells such as macrophages. The macrophage cells detect the bacteria through three groups of functional receptors and cause bacteria to be swallowed by connecting to them. In this process, three receptors are effective in the family of pseudo-TOLL receptors, each of which has been distinguished in identifying distinct parts of surface bacterial molecules. For example, 5TLR plays a role in detecting flagellin, 2TLR in identifying surface lipoproteins of bacteria and peptidoglycan, and ultimately 4TLR in identifying lipopolysaccharides. Lipopolysaccharide is present in the walls of gram-negative bacteria, including the Shigella bacteria, and leads to bio-activation in host by activating the inherent immune system. Its various components include the antigen O, the preserved central oligosaccharide and lipid A. Lipid A is a component of lipopolysaccharide that is known to be responsible for lipopolysaccharide biology activity and is called endotoxic agent, since it is the only part that is identified through contamination via 4TLR (5). For most lipopolysaccharide species, the TLR receptor serves as a particle recognition receptor or PRR, and recognizes lipopolysaccharide and initiates intracellular signaling trigger with an inflammatory response. The onset of a pro-inflammatory signal, by lipopolysaccharide, is related to the interaction between the 4TLR complex (14TLR4, MD2, CD) and lipid A in the lipopolysaccharide (6).

Pseudo-TOLL receptors are one of the most important receptors in inherent immune system. The most important task of these receptors is to identify the pathogen-accompanied molecules (7).

Pseudo-TOLL receptors, after being activated, trigger the production of cytokines and inflammatory chemokines and, on the other hand, produce antiviral responses and are considered as the most important receptors for inherent safety (8). Lipopolysaccharide of ligand gram-negative bacteria of the pseudo- TOLL receptor is 4 (4TLR) (9). The 4TLR is the most widely used recipients of the family, since it employs all the intracellular adapter proteins involved in TLR function, and on the other hand, it is the only TLR that produces cytokines and also generates anti-virus responses (7).

Mesenchyme stem cells are a group of mature stem cells that can be isolated from various sources such as bone marrow, fat tissue, wart gel in the umbilical cord, etc. Among these, mesenchyme stem cells derived from bone marrow have been the most com-

monly used specimen in experiments (10, 11). When bone marrow-derived and fat tissue-derived mesenchyme stem cells are stimulated with specific ligands from different TLRs, they activate NF-kB and signaling pathways of p13k and subsequently induce multiple genes and cytokines, in particular 10IL-6, CXCL and IL-8. Researches also show that mesenchyme stem cells clearly express TLRs. However, differences in the induction of genes have been reported in response to TLR activity. For example, Chu et al. and Tamchak et al. reported that effect of lipopolysaccharide, Escherichia coli and TNF-α, Poly IC and IL-β, respectively, are induced in bone marrow-derived and fat tissue-derived mesenchyme stem cells (12, 13).
Since the various strains of Shigella bacteria cause a dangerous disease in humans, it is essential to produce a good vaccine that can provide the maximum immunity in the body. Therefore, optimization of vaccine against Shigellosis disease was considered as a necessity in this research. Measurement of expression of pseudo-Toll receptors has previously been investigated between the two bacteria of Escherichia coli and Shigella Flexneri with an emphasis on expression of 4TLR in gram-negative bacteria (14), but, no comparison has been done between different strains of a gram-negative bacterium, especially in the stem cells and in order to produce the vaccine, this comparative trend has been considered as the goal of this study.

2. Materials and Methods

2.1 Preparation, Culturing and Bacterial Lysis

In this experimental study, different strains of used bacteria were prepared in Tehran University and confirmed by biochemical and serological methods (15-17). In order to produce lipopolysaccharide, each strain of Shigella bacterium was linearly cultured in tertiary agar culture medium (40 g/L) and placed in a temperature of 37°C for one day. Then, a single-colony was transferred to LB medium (25 g/L) and grown at a temperature of 37°C for one day at 150 rpm in a sugar incubator. After this time, tubes containing bacteria were transferred to centrifuge (8000 rpm for 5 minutes). After removing outer layer, the sediment was weighed, and then, based on the amount of sediment, a volume of lysis buffer was added to it which the concentration of each strain was ultimately reached to 0.1 gr/ml. Finally, the lysis of bacteria was carried out in 6 stages of 20 seconds and at rest intervals of 30 seconds by means of sonication, and the samples were transferred to the freezer at temperature of -20 ° C.

2.2 Culturing Mesenchyme Stem Cells Derived from Human Bone Marrow

Bone marrow-derived human mesenchyme stem cells, which were additional cells from individuals who referred to Royan Cell Therapy Center for osteoarthritis, were provided as frozen by Royan Research Center (Zaferaniyeh Center in Tehran). The identification stages of the above cells including distinguishing analysis and cell level markers were done previously (18) and after being received were cultured in the second passage.
Cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) with high concentration of glucose and 15% bovine serum to which penicillin/streptomycin antibiotics (1 µg/ml) were added. When the cells filled 90% of flask, they were trypsinized the cell count was performed at a concentration of 5×10 5 cells per cm2 and cellular passage was conducted. The cells were used in the third passage for further studies.

2.3 Cells Treatment with Bacterial Lysis

The cells in the third passage were transferred to the plate 6 of cell culture for separate treatments, and after 48 hours, they were treated at certain concentrations of 0.1, 0.01 and 0.001.
For completing cell treatment, the cells were transferred to a 37°C incubator at 5% CO2 concentration for one day. Three houses were considered for each concentration related to each strain. Finally, in order to reduce the error, the whole process of treatment was repeated twice. The cells were then trypsinized and transferred to microtubules to extract RNA.

2.4 RNA Extraction, cDNA Synthesis and Polymerase Chain Reaction (PCR)

Extraction of whole cell RNA was done by TRI method and samples were treated by DNase I to ensure that DNA is not contaminated. Then, cDNA synthesis was done by oligo-dT primer with reverse transcriptase enzyme MMuLV. 4TLR Primers were selected with Progressive Sequence: TGA TGT '5: and Reverse Sequence CTG CCT CGC GCC TG 3'
3' AAC CAC CTC CAC GCA GGG CT. Also, beta actin primers were designed by progressive sequence to confirm the synthesis of cDNA: CAA GAT CAT TGC TCC '5
5 'ATC CAC ATC: and Reverse Sequence TCC TG 3' TGC TGG AAGGs3'. The polymerase chain reaction was done with 4TLR primers and the cDNA synthesized in the previous step under the following conditions: initial denaturation for 5 minutes at 95 ° C, then 40 cycles of denaturation for 45 s at 95 ° C, spraying at 60°C for 45 s, extension at 72°C for 45 s, and finally, the final expansion at 72°C for 30 s. After terminating the process, PCR products were electrophoresed on an agarose gel with a concentration of 1.2% in order to be aware of the expression or non-expression of the samples.

2.5 Quantative Polymerase Chain Reactions (Q-PCR)

Polymerase chain reaction in real time was performed using synthesized cDNA, 4TLR primers and beta-actin. In all experiments, negative control was considered without the presence of cDNA. The main SYBR Green mixture of jump starter was added to each well of 96 PCR micro-plates (containing 10 µL SYBR Green, 6 µl water, 2 µl progressive and reverse primers and 2 µl cDNA) and PCR process was done in 50 cycles under 95°C for 30 seconds, 60°C for 30 seconds, and 72°C for 30 seconds. Finally, the data were analyzed using ANOVA statistical test (variance analysis of repeated intervals, split plot design).

3. Results and Discussions

3.1 Qualitative Expression of 4-TLR in Mesenchyme Stem Cells Treated by Cell Lysis of Shigella Strains

Expression of the 4TLR-related mRNA in mesenchyme stem cells was measured by the RT-PCR process. All treatments, except for Shigella sonnei Bacterium Lysis and Shigella dysentery, in the relative concentration of 0.001, showed 4TLR expression in human bone marrow-derived mesenchyme stem cells. No-treated mesenchyme stem cells that were considered as negative control did not show 4TLR expression (Fig. 1).

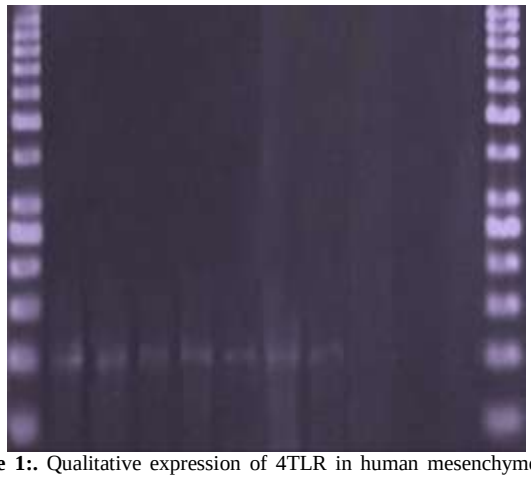

Figure 1:. Qualitative expression of 4TLR in human mesenchyme stem cells in treatment by lysis of different strains of Shigella bacterium. Wells 1 and 12: 50-bp molecular weight ladder; Well 2: Negative control (Mesenchyme stem cells without treatment); Wells 3, 4 and 5: Treatments with a relative concentration of 0.001 (respectively, sonnei treatments , Dysentery and Flexneri); Wells 6, 7 and 8: Treatments with a relative concentration of 0.01 (respectively, sonnei, Dysentery and Flexneri treatments); Wells 9, 10 and 11: Treatments with a relative concentration of 0.1 (respectively, sonnei, Dysentery and Flexneri treatments). Based on the results, no bond is observed in the wells of Shigella sonnei bacterial lysis and Shigella Dysentery at relative concentrations of 0.001 (respectively wells 3 and 4) and negative control (well 2). Based on the designing of the primer for 4TLR, the length of the given PCR product is 98 bp.

3.2 Comparison of 4-TLR Expression in Treated Groups

Three different technical replications were done for each experimental group of treatments. Analysis of polymerase chain reaction data in real time was performed using Applied Biosystem SDS7000 software. The required calculations were performed using ΔΔCT method. The data analysis was done using ANOVA statistical test (variance analysis of repeated intervals, split plot), and finally, a plot of the results was drawn. Based on the results obtained in all applied strains, the amount of expression level changed based on the dose, so that at the significant level of 0.01 ($p < 0.01$) in the cell lysis of all strains, the highest expression of the gene expression was related to the use of the dilution of 0.1 bacterial lysis on cell and the lowest expression of the gene was related to the use of dilution 0.001. According to the results and comparison of the effect of different strains on expression of 4TLR at a significant level of 0.01, the highest expression in human mesenchyme stem cells derived from bone marrow originated by the effect of cell lysis containing lipopolysaccharide of different strains of Shigella bacterium is related to Shigella Flexneri and the lowest expression level Sony was related to Shigella sonnei. However, no significant expression was observed in the negative control sample of the PBS-treated cells (Fig. 2).

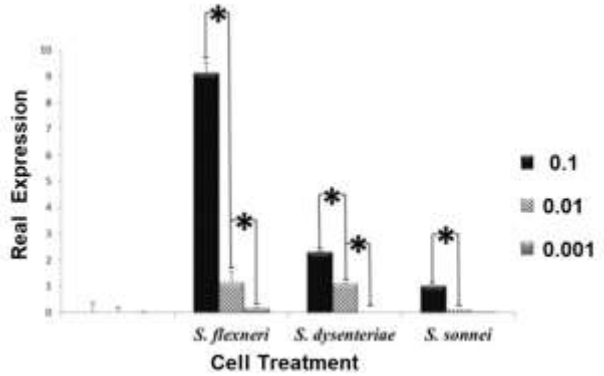

Figure 2:. Comparison of 4TLR expression in human mesenchyme stem cells in treatment with different strains of Shigella bacterium. The results are indicative of the dose-related expression. In all treatments, the highest expression of the gene was due to the use of dilution of 0.1 bacteria lysis on the cell and the lowest expression of the gene expression related to the dilution of 0.001 ($p > 0.01$). Also, the highest expression of 4TLR in human bone marrow mesenchyme stem cells obtained from the effect of Shigella Flexneri cell lysis and the lowest expression was related to Shigella sonnei. The negative control sample did not show any significant expression. Since the bacterial lysis was used as treatment, in order to ensure that only lipopolysaccharide in the lysis extract has led to the expresses of the mentioned gene, pure lipopolysaccharide of Escherichia coli were used as positive control. Results from the effect of Escherichia coli lipopolysaccharide at concentrations of 1, 10 and 100 µg/ml showed that at all three concentrations, the expression level of genes has significantly increased compared to the control sample, while at the concentration of 10 µg/ml gene expression was significantly higher than the other two concentrations ($p > 0.01$) (Fig. 3).

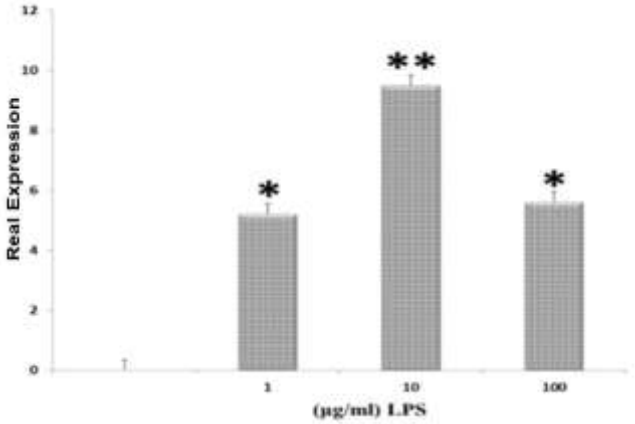

Figure 3:. Effect of pure LPS of Escherichia coli on 4TLR expression in mesenchyme stem cells. Development of nine positive controls including pure LPS of Escherichia coli at concentration of 1, 10 and 100 µg/ml showed that at all three concentrations, the expression of the gene was significantly higher than the control sample, while at a concentration of 10 µg/ml the amount of gene expression was significantly higher than the other two concentrations ($p > 0.01$) per ml.

4. Discussion

Many studies have been carried out by researchers to produce a vaccine against Shigella bacteria (17-19). In this study, the immune-stimulation rate was considered based on TLR expression. Since lipopolysaccharide of negative gram bacterium like Shigella ligand is a 4TLR, thus, comparative evaluation of stimulation of this receptor via lipopolysaccharide of different strains of Shigella bacteria was considered the main objective of the present study in order to identify suitable lipopolysaccharide of this species in stimulating the immune system and producing the vaccine.

As in this study, cell lysis containing lipopolysaccharide was used as the treatment, the highest stimulation was related to lysis of Shigella Flexneri bacteria and the lowest stimulation was related to Shigella sonnei. In addition, in all treatments, the amount of expression varied depending on the dose. According to the results, it was found that not only the type and source of lipopolysaccharide, but also its level is effective in stimulation and expression of 4TLR. Prior to this study, many researchers studied the effect of lipopolysaccharide on the expression of 4TLR and its subsequent cascade pathway and in each of these studies, lipopolysaccharides of various species were compared with each other (6, 19), but in this study, for the first time, the comparison between different strains of a species was done to produce the optimal vaccine. Additionally, in above study, bone marrow-derived human mesenchyme stem cells were used as the basis for experimental study. The reason for the selection of this cell class, in addition to their rapid growth, was that these cells are among the closest cells to their immune cells in their nuclei, and this is perhaps the reason why in various studies the basal level of expression of TLRs has been shown in the cell (20, 21). This has led to the activation of

these receptors in these cells as an important factor in determining the function and characteristics of these cells (22, 23).

The bacterial lipopolysaccharide is very complex combination and its structural composition varies greatly with the different species of gram-negative bacteria. Structural variations such as the amount of anisolation, the length of the acyl chains, and the degree of phosphorylation of lipid A that affect the signaling (24, 25) alter the interaction of these molecules with the 4TLR receptor. The number of fatty acid chain is a major contributor to the immunizing of endotoxin of the endotoxin. The most active form of lipid A is the 6- chain of fatty acid found in Escherichia coli and Salmonella species. Lipid A containing less acyl, e.g., four and five acyls, induce little host defensive domains (26). In a study in which both lipopolysaccharides of Shigella Flexneri Escherichia coli were used, the stimulatory activity of lipopolysaccharides Flexneri was lower compared to lipopolysaccharides at similar concentrations of lipopolysaccharides. These differences confirmed the previous observations based on the fact that lipopolysaccharides containing six 6-acyl lipid (e.g., Escherichia coli) exhibit more stimulating characteristic than lipopolysaccharides that Lipid A with lower acyl chain e.g., five or four acyls, has been applied in producing them (e.g., Lipopolysaccharide Shigella Flexneri A2) (26, 27). However, the identical transference of both lipopolysaccharides of Escherichia coli and Shigella Flexneri showed luciferase activity derived from NF-kB at significance level. Kim et al. (27) in their study reported the difference between lipid A hexa-acyl and penta-acyl in terms of their efficacy for the accumulation of 2TLR4 / MD complexes. According to studies by Hatryl et al., Shigella, serotype A5 often has lipid A hexa-acyl (93%), in contrast to Shigella Flexneri A2 in which Pentacyl content is higher. Given that the ratio of pentazole of all strains used was higher, it is likely that the reason of this result is another factor other than the number of acyl chains in lipid A, or that it can be said that the lipid A ratio penta-acyl is higher than tetra- and tri-acyl in Lipid A of Shigella Flexneri compared to other strains and this confirms the results of other studies in this area.

Huber et al. (28) recently in a study that compared lipopolysaccharide of Salmonella and Escherichia coli reported that a rough form of LPS derived from Salmonella Minnesota or Escherichia coli is more powerful than its smooth form showing that in addition to Changes in lipid A, LPS core sugars, or repetitive O antigens are also involved in 4TLR in response to lipopolysaccharide. In addition, they showed that neither lipid A nor rough lipopolysaccharide needed no 14CD or LBP for signaling, while this is not so for smooth lipopolysaccharides. However, Henrykson et al. (29) showed that Lipid A or lipopolysaccharide with a rough mutation has a less stimulating property than a smooth lipopolysaccharide. The reason of this contradiction is unknown and may be due to the varying solubility of lipopolysaccharide or in vitro (conditions such as the presence and absence of the culture medium).

Other studies showed that the high 2-MD increases lipopolysaccharide signaling by 4TLR (30). This suggests that high levels of humanMD-2 are needed in addition to high concentrations of lipid A to induce the activity of this system through lipid A. Due to the same conditions of the culture medium for all specimens, the reason for the difference in expression at the transcriptional level and the expression of 4TLR is probably due to differences in the length of acyl chain, degree of phosphorylation of lipid A, or even the amount of sugar in the antigenic structure of O is the structure of lipopolysaccharide. The results of this test and the fact that Shigella Flexneri A2 has the highest gene expression level and 4THL and thus the highest immune stimulation rate, confirms the others' data on the designing of the vaccine against Shigella definitively.

Due to the high risk of pathogenicity in working with Shigella strains and their cellular extracts containing many immunologic agents, which is considered as a limitation of this study, therefore, based on the results and comparing them with the results of other reports is suggested that in this study, the lipopolysaccharide of the above strains, the process of stimulating the immune system and the expression of 4TLR should be studied more accurately as well as the dependent production and secretion of cytokines and chemokines. Similarly, according to past reports about high Lipid at hexa-acyl levels in Shigella Flexneri A5 than A2, in another study comparing the immunogenicity of lysis of these two types of Shigella Flexneri also seems interesting. On the other hand, considering that in producing Invaplex vaccines an IpaC, IpaB, and optimal lipopolysaccharides of bacterial strains of a species are used, it is suggested that to apply Flexneri strain lipopolysaccharide for the production of Invaplex vaccine against sonnei, dysentery and bouillon strains (due to more immunogenic power). In addition, because TLR signaling modifies and regulates the function and characteristics of mesenchyme stem cells, and considering the widespread and important use of these cells in the cell therapy of autoimmune diseases, the evaluation of lipopolysaccharide effects on the functional process and changing of the characteristics of these cells is also considered as an attractive idea.

5. Conclusion

Intrinsic immune responses are induced by lipopolysaccharides of gram-negative bacteria as a 4TLR agonist. The combination of lipopolysaccharide in various species of gram-negative bacteria is very variable. Therefore, expression of different levels of 4TLR is expected due to stimulation by different strains of Shigella bacteria. The results from this study, in addition to confirming this, showed that the most suitable lipopolysaccharide of Shigella strains for producing the vaccine is Flexneri strain of lipopolysaccharide which results in the highest expression of 4TLR. In addition, due to the concentration results, the optimal concentration used by Flexneri lysis can be considered as the appropriate dose for vaccine preparation.

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