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The effect of recombinant FlgE2 protein from *Helicobacter pylori* on nitric oxide production in macrophages

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ABSTRACT

Helicobacter pylori is a human pathogen that lives permanently in the stomach. The immunomodulatory effects of some protein fractions of the bacterium have been suggested as an immune stimulus factor in the design for a vaccine. *Helicobacter pylori* FlgE2 protein is a component of bacterial flagellum whose effects on innate immune cells have not been studied in depth. In the present study, we aimed to assess the effect of FlgE2 on the production of nitric oxide (NO) by rat peritoneal macrophages.

Recombinant *Helicobacter pylori* FlgE2 protein was used. Peritoneal macrophages of mice were removed and cultured. Different concentrations of recombinant protein were used to stimulate macrophages and assess NO production. To detect NO, macrophage culture supernatant was removed and evaluated by reagent grease. Finally, the results were evaluated by SPSS software. The results showed that the recombinant FlgE2 protein from *Helicobacter pylori* increased the level of nitric oxide by increasing concentration. Nitric oxide level was measured at concentrations of 4 µg/ml, 20 µg/ml, 40 µg/ml and 80 4 µg/ml. The highest level of production was observed at 80 µg/ml (P=0.01). In the last condition NO was significantly larger compared to the LPS control group.

According to the findings of this study, recombinant FlgE2 has a positive effect on stimulation of NO production by peritoneal macrophages. Therefore, it is suggested that recombinant FlgE2 can be proposed as an immunostimulant for vaccine candidates.

Keywords: FlgE2 recombinant protein, Macrophages, *Helicobacter pylori*.

Introduction

Helicobacter pylori (*H. pylori*) is a specific human stomach pathogen that colonizes the stomachs of about half of the world's population. Its prevalence is estimated at more than 80% in middle-aged adults in many developing countries, while it is 20-50% in industrialized and developed countries. Infection is caused by oral ingestion of bacteria, and its transmission occurs primarily among family members and in early childhood (1, 2). The majority of patients are asymptomatic; however, infection in some of them is associated with the development of peptic ulcers, gastric adenocarcinoma, gastric MALT lymphoma, and non-Hodgkin's lymphoma (3,4). *H. pylori* is a bacterial flagellum making the basis of bacteria viability in the human stomach. FlgE protein is a part of the hook in the bacterial flagellum, which causes a flexible bond at the surface of the stomach cell (5-7). In *H. pylori* genome there are two genes at different positions, known to code for FlgE proteins, FlgE1 and FlgE2, that are components of the helix hook. *H. pylori* FlgE2 is composed of 605 amino acids, for a molecular weight of 66 kDa. The hp0908 gene encodes the FlgE2 protein and is located inside the operon containing the hp0906 and hp0907 genes that encode the FliK and FlgD proteins, respectively. These proteins are involved in regulating hook length. The hp0870 gene, on the other hand, encodes the FlgE1 protein and is located inside an operon containing two different regulatory genes, including hypA, which encodes nickel hydrogenase, and lpxB, which is a *lipid A disaccharide synthase*. Like most bacterial flagellum proteins, FlgE2 presents an antibacterial recognigion site by exposing H-antigens at the front domain. This feature makes the protein a potential target for a vaccine (8). The immune response to *H. pylori* is involved in the pathogenicity of this bacterium. There is evidence that this bacterium cannot be accessed by the immune system through a variety of mechanisms, but the complex reaction of *H. pylori* and the innate and acquired immune systems still remain unknown (9). Macrophages are the central mediators between the innate and acquired immune systems as well as the predominantly onset of the immune response against *H. pylori*. The pathways of the immune response to Th1 or Th2 are determined by the cytokines produced by macrophages (10). Nitric oxide (NO) is a molecule that has recently attracted a lot of attention due to its wide-ranging role in human health and biology, and it is important in macrophages (24). The role of NO in the immune system is extensive, and while much is known about some specific roles of NO, it's behavior outside the norm is still largely unknown. The well-known role of NO in the immune system is anti-tumor and anti-microbial effects. NO derived from macrophages can induce senescence or death of tumor cells (25,26). Cytotoxic lymphocyte cells can also induce the expression of NO in tumor cells through interferon-γ (IFN-γ) and tumor necrosis factor (TNF), which again leads to senility or death (25,27). Some compounds such as IFN-γ, single or in with microbial products [e.g. lipopolysaccharide (LPS) or cytokines [e.g. tumor necrosis factor (TNF)], can cause macrophage activation (28). Classical macrophage activation is characterized by a high capacity for antigen presentation. High production level of interleukin 12 (IL-12) and IL-23 is effective (29), and it causes the activation of a polarized type I response, and the production of toxic mediators [nitric oxide (NO), reactive oxygen species (ROI)] increases. Many have referred to these cells as M1 macrophages, mirroring the Th1 nomenclature (30,31). Several studies have examined the reactions of *H. pylori* and macrophages (11); meanwhile, research on the reaction of individual pathogens with these cells can reveal obscure aspects of the pathogenesis of this bacterium. Over the recent years, studies have examined the reaction between certain pathogens, such as CagA and VacA, with macrophage cells. The aim of this study is to the effect of recombinant FlgE2 protein from *H. pylori* on nitric oxide production in macrophages.

Materials and Methods:

1. Purification and expression of recombinant protein FlgE2 protein:

FlgE2 recombinant protein was produced in the Padova university lab according to the procedure decribed (8,12) and then transferred to Iran. Briefly, the FlgE2 gene was amplified, cloned and expressed with a hexa-histidine tag at the C-

terminus in *E. coli* BL21 (DE3) cells and purified as described (8).

2. Evaluation of recombinant protein by SDS-PAGE:

In order to analyze the recombinant protein produced, SDS-PAGE electrophoresis method with a concentration of 12.5% polyacrylamide gel and a voltage of 100 V was used in the presence of protein marker ladder. Finally, the polyacrylamide gel was stained with coomassie blue G-250 and examined in Shahid Beheshti University lab.

3. Isolation, culture, and stimulation of macrophages

Female 6-8 week old BALB/c mice were purchased from Pasteur Institute of Iran. To isolate peritoneal macrophages, 10 ml of cold RPMI-1640 (Sigma) culture medium were injected into mice peritoneum. Macrophages were separated from 5 mice and mixed together. Then cell seeding was done after washing and counting cells, in each well $1.5*10^6$ per/ml cell was seen. Cells in each well were in RPMI medium containing supplemental form; 2 g/l sodium bicarbonate, 2 mM L-glutamine, 100 units/ml penicillin, 100 µg/ml streptomycin (Merk), and 10% fetal bovine serum (Sigma), and 200 µl of this suspension in each plate 96 houses (Nunc, Denmark) was cultured for 4 hours at 37 ° C and 5% CO2. During incubation, adherent cells (95% macrophages) adhered to the bottom of the plate (13). After incubation, non-adherent cells were washed three times with RPMI at 37°C . Recombinant FlgE2 protein was used to stimulate macrophages in a series of concentrations of 500, 50, 5, 0.5, 0.5, 0.05, and 0.005 µg/ml. Each antigen concentration was added in triplicate to 96-well plate cultures of macrophages and a group of lipopolysaccharide-stimulated macrophage cells (LPS) as control was cultured (16).

4. Nitrite concentration measurement

During macrophage culture, nitric oxide into the culture medium was released. Nitric oxide is unstable and is rapidly converted to nitrite and nitrate (14). As a result, the amount of nitrite in the culture medium can be measured according to the method of Nathan and Stover (17). In this method, a Griess reagent was used. After 48 hours of macrophage culture, the culture supernatant was removed and mixed 1:1 with a grease reagent in a 96-well plate (samples were tested in triplicate). After 15 minutes, the absorbance (OD) at 540 nm by using a microplate absorption reading device (Multi Scan) was measured. To calculate nitrite concentration, standard diagram (1-200 mM) from sodium nitrite solution (NaNO2) was used (15,18).

Statistical analysis

This study was an interventional study. In this study, categorical variables were presented as numbers (%) and continuous variables as mean ± SD. The one-way analysis of variance (ANOVA) followed by Tukey test was used to assess indifferent concentrations of recombinant protein. Statistical analyses were conducted using the IBM SPSS Statistics for Windows, Version 22.0 (IBM Crop., Armonk, NY, USA) and Prism6 software. All statistical tests were 2-tailed, and a $P<0.05$ was considered statistically significant.

Results:

1. Expression and purification of recombinant FlgE2 protein

According to the report of researchers from Padova lab, a Full-length form of FlgE2 with His6 tag flanked to the C-terminus was successfully cloned and expressed in a heterologous expression system. The protein was purified in two different oligomeric forms. In order to avoid buried surfaces generated by its tetrameric assembly, only the monomeric form was further concentrated and used for cell culture. The protein counts 605 amino acids and proteins with a molecular weight of about 66 kDa.

Expression and purification of recombinant FlgE2 protein.

SDS-PAGE showed the expression of a protein within the 66 kDa band of the weight guide, which is consistent with the predicted molecular weight of about 66,000 daltons (Figure 1).

Based on measurements by Bradford method (with BSA standard) and spectrophotometry, sediment protein extraction from one liter of bacterial culture was determined to be about 7 mg.

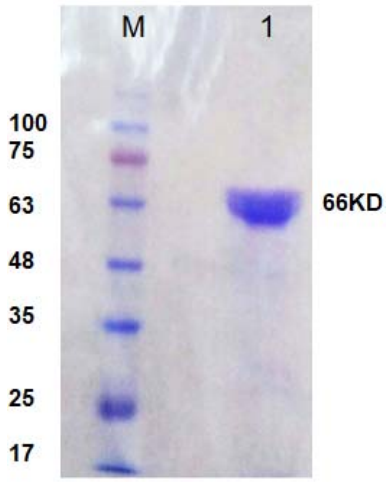

Figure 1: Evaluation of FlgE2 protein expression in SDS-PAGE gel (12%): (1): FlgE2 protein (diluted 10-fold), (M): Ledder

2. Results of nitric oxide production from macrophages:

The amounts of nitric oxide produced by macrophages in the presence of different concentrations of Hp-FlgE2 are illustrated in Figure 2. The results showed that the production of nitric oxide at a concentration of 80 µg/ml of recombinant FlgE2 protein was significantly higher compared to the untreated control group and on the other hand, at a concentration of 80 µg/ml compared to the group Positive control treated with LPS has a significantly higher level (P<0.001).

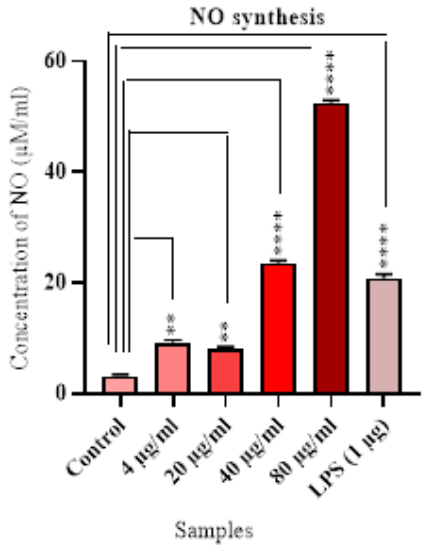

Figure 2: Measurement of nitric oxide produced by macrophages in treatment with different concentrations of Hp-FlgE2

Statistical analysis showed that the rate of stimulation of nitric oxide production at a concentration of 40 µg/ml was also higher than the LPS positive control group (P<0.001 for both). In the instrumental values of 20 and 4 µg/ml, no significant difference was observed between the test group and the positive control of LPS.

Discussion

The importance of investigating *H. pylori* is in view of the prevalence of this pathogen in 50% of the world's population and the development of very important diseases, such as chronic gastritis, gastric ulcer, and gastric adenocarcinoma. *H. pylori* can produce superficial compounds that are chemotactic to neutrophils and monocytes, which are in turn involved in epithelial cell damage and inflammation (19). No vaccine has been produced against *H. pylori* to date, and it is important to examine the signaling pathways to be able to suggest a vaccine candidate. Furthermore, no study has been conducted on protein FlgE2. It has been done for the first time in Iran and the world. This study is a reconstruction of the immune response process in vitro. The results could suggest an investigation of the antigenicity of candidates for vaccination purposes.

FlgE2 antigens are the outermost part of this pathogen, the antibody against which is known as an antibacterial diagnostic site that has made it a potential target for vaccines for immunological treatment; hence, understanding its structure is of particular importance. The structure and function of the flagellum are critical in this pathogen. *H. pylori* flagella are influential factors in colonization due to inflammation and escape from the immune system (20).

Jinn-Jong et al. (2016) studied the effectiveness of FlaA and r-FlgK proteins of bacterial flagellum on the serological response of Balb/c rats. The results indicated that treatment with these protein could strongly stimulate the immune system and antibody production.

According to an article by Waskito et al. (2018), *H. pylori* flagella in epithelial cells increased the secretion of inflammatory cytokines, including IL-6, IL-8, and TNF-α, which stimulated and activated the immune system to produce *anti- H. pylori* responses (19).

These responses suggest the selection of a more specific candidate, such as FlgE2.

Nitric oxide acts as an effective molecule and physiological messenger in many biological systems, including immune tissues. It also affects the development of the appropriate acquired immune responses against bacteria and the nervous and cardiovascular systems, and is a mediator in various diseases, including vascular diseases, diabetes, renal ischemia, cancer, and inflammatory diseases. On account of the role of NO in the above-mentioned systems, its measurement is very important in tissues and biological fluids.

Previous studies have suggested that nitric oxide (NO) is a free radical molecule produced through interaction with iron-containing proteins, such as soluble guanylate cyclase (sGC), or forms protein additives, containing nitrogen oxide functional groups (S-nitrosothiols, 3-nitrotyrosine, and dinitrosyliron complexes). It has been also shown to play a signaling role. These interactions lead to several different effects and functions in physiology and diseases. There is considerable empirical evidence concerning the establishment of NO as an endogenous epigenetic regulator in gene expression and cell phenotype. Nitric oxide has been reported to affect the key aspects of epigenetic regulation, including post-translational histone changes, DNA methylation, and microRNA levels. Disease background, epigenetic protein regulation, and NO-mediated enzymatic activity have been also investigated. Therefore, the effect of this factor on the carcinogenicity of the *H. pylori* pathogen is of great importance. The present study examined the amount of nitric oxide and its concentration, which increased in general and depended on the concentration in the treated cells. According to the results, the amount of NO was 52.0904 at a concentration of 80 µg/ml of recombinant protein, indicating a high concentration of NO in macrophage cells. It can be argued that the recombinant protein could be a risk factor for cancer cells due to the changes in the morphology and structure of DNA. These results are consistent with those obtained in a study by Socco et al. in 2017 (20).

The results of this study indicated that the presence of different concentrations of recombinant protein increased the expression of nitric oxide that played a role in regulating the secretion of inflammatory cytokines.

There is no research on the effects of recombinant FlgE2 protein on the THP cell line. According to a study by Chen et al. (2017), inflammation is an innate immune response leading to host protection. However, the proinflammatory agents, ROS, NO, and other biologically active substances are released after persistent infection, that together with the host response to "excessive inflammation", double tissue damage (21). In this study, the recombinant *H. pylori* protein also raised NO expression, which was consistent with the hypotheses. Based on the results of ELISA test by Chen et al. on LAMP derivatives in the Mycoplasma pneumonia pathogen, NO, as an important inflammatory mediator in the cell culture supernatant, was measured in human macrophage cells treated with this structure (22). The structure of LAMP increased the concentration of NO in the supernatant, leading to an increase in the concentration of NO in the culture of macrophage cells treated with recombinant FlgE2 protein, and its concentration increased by five times at 80 µg/ml compared to the positive control of LPS. This indicates that this recombinant protein stimulates the immune system and is a good candidate for the production of vaccines. The results obtained in this research also revealed that the derivatives of this pathogen are effective in cell function. Furthermore, treatment with endotoxin contributes to higher levels of expression of cellular mediators, such as nitric oxide, in immune cells.

Conclusion

According to the findings of this study, recombinant FlgE2 has a positive effect on stimulation of Nitric oxide production by macrophages. Therefore, it is suggested that recombinant FlgE2 can be proposed as an immunostimulant for vaccine candidates. Bacterial secondary products with different effects on immune cells are a perfect candidate for a suitable response for prevention and vaccination. So animal experiments can help achieve this ultimate goal.

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