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Extracellular Nucleic Acids Secreted by *Lactobacillus* Regulate TLR9 Expression

Chun-Liang Liu, Shu Wen, Yin-Hui Liu, Hua-Jun Li, Jing Xiao and Li Tang

Department of Microecology, Dalian Medical University, 9 West Lvshun Southern Road 116044 Dalian, China

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Abstract: Objective: The study was designed to determine the immune mechanism of nucleic acid sequences present in the medium filtrate of *Lactobacillus* DM8909 at exponential growth phase. The effect of extracellular nucleic acids on TLR9 on the expression of IL-6 was also investigated.

Methods: Three groups were formed as nucleic acid group, CpG-ODN group and control group. Extracellular nucleic acids were examined by TA cloning and sequencing technology. RT-PCR and ELISA were used to detect the expressions of TLR9 and NF- κ Bp65 in HT-29 cells and the presence of IL-6 in the medium.

Results: The nucleic acids detected in the medium filtrate of *Lactobacillus* DM8909 at exponential growth phase were found to be RNAs. A total of 39 RNA sequences were identified as *Lactobacillus delbrueckii* subsp. *Bulgaricus* with a size of 49 bp, 23S ribosomal RNA and another RNA with a size of 52 bp identified as *Lactobacillus rhamnosus*, ATCC 53103, tRNA-Leu sequence. TLR9 expressions in CpG-ODN and nucleic acid groups were higher compared to the control group (TLR-9 values were 3.2386, 0.4984 and 0.1780 respectively). The same results were obtained for NF- κ Bp65 where the expressions in CpG-ODN and nucleic acid groups were higher than the control group (relative values were 1.6671, 0.6501 and 0.3094 respectively). Furthermore, IL-6 expressions in CpG-ODN and nucleic acid groups were significantly higher than the control group ($P < 0.05$, (relative values were 23.6735 ± 1.4515 , 20.2041 ± 1.6820 and 15.7143 ± 0.7047 respectively).

Conclusion: The extracellular nucleic acids in the medium of *Lactobacillus* DM8909 are RNAs and they could elevate the level of IL-6 through the activation of NF- κ B and TLR9 *in vitro*. These molecules have similar immune activity with CpG-ODN.

Key words: *Lactobacillus*, RNA, TLR9, CpG-ODN, NF- κ Bp65, IL-6

Introduction

Lactobacillus is present in a wide range in human gastrointestinal tract and vagina. They play important roles like maintaining intestinal and vaginal self-stabilization, promoting maturation of the immune system and improving the immunity of the hosts^{1, 2}. *Lactobacillus* DM8909 was isolated in a healthy woman's vagina in 1989 by our research team. It is a *Lactobacillus delbrueckii*, which has been used to treat bacterial vaginosis in China. In 1999, its clinical use was approved by the Chinese Ministry of Health³. *Lactobacilli* DM8909 used in the treatment of bacterial vaginosis obtained a success rate of 87.1% (54/62) and an improvement rate of 91.9% (57/62). However, its immune mechanism against infection is still unknown. Nucleic acids about 100 bp were found in the medium of *Lactobacillus* DM8909 at exponential growth phase but the composition, properties,

sequence and immunological activity are not very clear. A recent study revealed that that cytosine-phosphate-guanosine oligodeoxynucleotide (CpG-ODN), an extracellular nucleic acid substance plays an important role in mediating NF- κ Bp65 through TLR9, in order to accommodate the cytokine and performs its role in the immune system^{4, 5}. We explored the nucleic acid's immune mechanism via the effect of nucleic acids on TLR9-mediated immune pathway.

Materials and Methods

Bacterial strains and culture

Lactobacillus DM8909 was grown in the Department of Microecology, Dalian Medical University. *Lactobacillus* DM8909 was cultured in conventional LBS culture medium.

Extraction and identification of nucleic acids

A total of 500 ml of the medium where *Lactobacillus* DM8909 at exponential growth phase was collected and filtered by G6

Correspondence to: Dr. Li Tang, Institute of Microecology, Dalian Medical University, Dalian, 116044 • CPR China. Tel.: +86-411-8611-0152; Fax: +86-411-8611-0152. E-mail: tangli1484@sina.com

Table 1. Primer synthesis and RT-PCR reaction conditions

Targeting gene	Primer	Fragments length	Annealing temperature
TLR9	5'-GCA CAG GAG CGG TGA AGG T-3' 5'-GCA GGG GTG CTC AGT GGA G-3'	820bp	63
NF-κBp65	5'-ATG TGC ATC GGC AAG TGG -3' 5'-TGC TGG GAA GGT GTA GGG -3'	332bp	61
β-actin	5'-TCT ACG AGG GCT ATG CTC TCC-3' 5'-GGA TGC CAC AGG ATT CCA TAC-3'	320bp	58

bacteria filter. An equal volume of pre-cooled ethanol was added into this filtrate. After shaking, the filtrate was centrifuged at 12,000 rpm at 4°C for 20 min. The precipitate was dissolved with DEPC water and 0.2% chloroform was added and thoroughly mixed.

Samples were set it in the ice box for 5 min, centrifuged at 12,000 rpm at 4°C for 15 min and the supernatant was collected. A volume of 0.6% isopropanol was added into the solution and was placed inside the ice box for 10 min. The solution was then centrifuged at 12,000 rpm at 4°C for 10 min. The precipitate was collected and 75% ethanol was added and centrifuged at 7500 rpm 4°C for 5 min. The sediment was washed with DEPC water and quantitative detection was done by UV spectrophotometer at A260. A solution of 10 μL solution was digested for 15 min with DNAase without RNAase and checked in 2% gel electrophoresis. Extracted RNAs were stored at -80°C freezer, for sequence analysis and biological experiments.

Sequence analysis of RNAs

Sequence analysis of RNAs was performed at Shenzhen HuaDa Genetics Technology Co., Ltd. (www.genomics.cn). Briefly, 100 ± 50 bp of RNA fragments by gel electrophoresis were purified and RNAs about 200-700 bp were made and connected by solexa joints to run the reverse transcription in the 3' and 5' end followed by PCR amplification. pMD18-T Vector kit was used to do TA cloning of PCR products. After cloning, BEST™ Sequencing Primers and M13 Primers were used to sequence the DNA (ABI3730 sequencing Instrument). Target sequences were separated by chrome software in peak sequencing and were analyzed one by one with Multalin multiple sequence alignment software online (<http://multalin.toulouse.inra.fr/multalin/multalin.html>, <http://blast.ncbi.nlm.nih.gov/Blast.cgi>, <http://www.genomics.cn/index.php>).

RT-PCR analysis of the expression of TLR9 and NF-κBp65 in HT-29 cells

Cells were divided into three groups: nucleic acid group, CpG-ODN group and control group. Each group contains 300 μL of HT-29 cells at a concentration of 1.0×10^6 /mL. Nucleic acids and CpG-ODN were added to their respective group to reach a final concentration of 1.0 μM. Medium was added to the control group. The 3 groups were placed in incubator at 37°C with 5% CO₂ for 48h. The cells were then collected, washed 3 times with cold PBS before total RNA extraction.

Primer synthesis and RT-PCR detection

The primers of the target genes NF-κBp65 and TLR9 were synthesized by Takara Biotechnology (Dalian) Co., Ltd. The primer volume for amplification of TLR9 and NF-κBp65 in nucleic acid group was C2 = 1μg/μL, in CPG-ODN group was C3 = 1μg/μL, in the control group was C1 = 1μg/μL. The reaction conditions were as follows: pre-denaturing at 94°C for 2 min and annealing for 30 sec with temperature determined by the primers designed (Table 1). When the reaction was completed, 5 mL reaction products were viewed at 1.5% agarose gel electrophoresis. The electrophoresis buffer was TAE with a voltage of 80V for approximately 40 min. The bands were analyzed by Quantity One electrophoresis gel imaging. Relative quantitative analysis of the bands was compared with β-actin to calculate the relative amounts of mRNA expression of TLR9 and NF-κBp65.

IL-6 levels in HT-29 cell by ELISA

HT-29 cells with a count of 1.0×10^6 /mL each were divided into three groups. They were inoculated into 6-well plates in duplicates and cultured at 37°C in 5% CO₂ incubation chamber. After confluence, nucleic acid and CpG-ODN were added into the corresponding wells to reach the final concentration of 1.0 μM and then cultured for 48 h. Then after, the culture medium was collected, centrifuged at 2000 rpm for 15min and the supernatant was transferred into sterile EP tubes. Quantitative measurement of IL-6 was done with ELISA following the manufacturer's protocol.

Figure1. The characteristics of the product extracted from the culture filtrate of *Lactobacillus DM8909* exponential growth phase. (A) The figure was performed by Takara Biotechnology (Dalian) Co., Ltd.. M: DNA Marker D2000, S: Sample. (B) The composition analysis of the product. Lane 1: DL2000 Marker, Lane 2: Sample, Lane 3: Sample-treated with RNase, Lane 4: Negative control (LBS not fermented). (C) The degradation of nucleic acids which was preserved in liquid nitrogen for approximately one month. Lane 1,2: New extracted nucleic acids, Lane 3: DNA Marker D2000, Lane 4,5: Nucleic acids preserved in liquid nitrogen for approximately one month.

Figure 2. The growth curve of *Lactobacillus DM8909* (A) and the OD260 of the product extracted from its culture filtrate (B). *Lactobacillus DM8909* was twice proliferated in LBS broth for 24h before inoculated into the detected system (large volume LBS broth). From Fig. 1 (A), the exponential growth phase of *Lactobacillus DM8909* was approximately 6 to 14 h. According to the growth curve of *Lactobacillus DM8909*, the OD260 value of the product extracted from the culture filtrate of *lact*

Statistical analysis

SPSS13.0 software was used for all computations. T-test was used as indicated by $\bar{x} \pm S$. A P-value of <0.05 was considered significant.

Results

Extraction and identification of nucleic acids

Electrophoresis of the products from the culture filtrate of *Lactobacillus DM8909* at exponential growth phase revealed the existence of a large number of 100 bp nucleic acids (Fig. 1A). After the treatment of DNase (Free RNase), 50-100 bp RNAs were found (Fig. 1B). Excretion of RNAs at the exponential growth phase medium closely paralleled with the growth of bacterial counts showing a significant positive correlation (Fig. 2, Table

Fig 3. RNA abundance

Fig.4 RNA sequencing map

Table 2.Solexa adapter sequences and PCR primers

Solexa adapter sequences	PE Adapter1.1 (32 bp)
	5' P- GATCGGAAGAGCGGTTCAGCAGGAATGCCGAG
	PE Adapter2.1 (33 bp)
PCR primers	5'ACACTCTTTCCCTACACGACGCTCTTCCGATCT
	PE PCR Primer1.1 (58 bp)
	5'AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCTTCCGATCT
	PE PCR Primer2.1 (61 bp)
	5'CAAGCAGAAGACGGCATACGAGATCGGTCTCGGCATTCCTGCTGAACCGCTCTTCCGATCT

2). The growth of *lactobacilli* reached a peak after 12 h at the exponential growth phase and the generation time of *lactobacillus DM8909* is 1.95 h. Figure 3 shows RNA abundance.

Sequence analysis of RNAs

TA cloning of the medium filtrate of *lactobacillus DM8909* at exponential growth phase obtained 39 RNAs sequences. Two of

Figure 5. Electrophoresis analysis the expression of TLR9, NF-κBp65 in HT-29 cells treated with nucleic acids

them were absolutely clear, one was 49 bp with a sequence of CGTGAAATCCCGTTTGAATCCGCGAGGACCATCTCGCA AGGCTAAATAC identified as *lactobacillus delbrueckii subsp. Bulgaricus*. The other one is 23S ribosomal RNA and another was 52 bp with a sequence of CCACGGTCAAAAACCACTAGA ACCTAAATCTAGCGGTCTGCCAGTTCCGCC identified as *lactobacillus rhamnosus*, ATCC 53103 tRNA –Leu (Fig. 4).

RT-PCR analysis of the expression of TLR9 and NF-κBp65 in HT-29 cells

Our research showed that the nucleic acids affected the expression of TLR9 and NF-κBp65 in HT-29 cells compared to the control group. TLR9 is higher in the nucleic acid group than the control group but lower than the CpG-ODN group (TLR-9 expression is 0.1780 in the control group, 0.4984 in the nucleic acid group and 3.2386 in CpG-ODN group). Likewise, NF-κBp65 expression is highest in CpG-ODN group followed by the nucleic acid and then the control group. (NF-κBp65 expression is 0.3094 in the control group, 0.6501 in nucleic acids group and 1.6671 in CpG-ODN group) (Fig. 5 and Fig. 6).

IL-6 levels in HT-29 cells culture medium by ELISA

The experiments showed that nucleic acids had an impact on the IL-6 levels in HT-29 cells compared to the control group. IL-6 expression is significantly higher in the nucleic acid group

Table 3. IL-6 levels in the culture medium of HT-29 cells affected by nucleic acid components

Group	N	IL-6 ug /g ($\bar{x} \pm s$)
Control group	7	15.7143±0.7047 ^{i±}
Nucleic acid group	7	20.2041±1.6820* ^{i±}
CPG-ODN group	7	23.6735±1.4515*

Note: * p <0.05 compared with the control group;

³p <0.05 compared with CPG-ODN intervention group group

Figure 6. Relative value of TLR-9, NF-κBp65 in HT-29 cells treated with nucleic acids

secreted than in the control group but significantly lower than the CpG-ODN group (control group is 15.7143 ± 0.7047 , nucleic acid group is 20.2041 ± 1.6820 , CpG-ODN group is 23.6735 ± 1.4515) (Fig. 7 and Table 3).

Discussion

There are over a thousand kinds of bacterial flora in the human intestine with total number of more than 1 trillion. They mainly establish colonies in the colon, forming a complex ecosystem with a wide range of bioactivity affecting the human's intestinal flora and normal functions. This phenomenon is closely related to the occurrence and development of intestinal diseases, particularly chronic infectious intestinal diseases ^(6, 7, 8). Some metabolites of intestinal flora play an important role in this process, such as p40 and p75 proteins secreted by *L.rhamnosus* GG where in they could promote the growth and prevent tissue damage by excessive growth of TNF-α⁽⁹⁾. The metabolites which regulate the intestinal immune system and the function of immune cells directly are not very clear and little is known about the mechanism how metabolites influence the occurrence and development of chronic infectious intestinal diseases. TLRs are co-stimulatory receptors of foreign antigens which play an important role in identifying bacteria, regulating the immune responses induced by antimicrobial peptides and in removing pathogens. TLR9 expression on the apical and lateral intestinal epithelial basement membrane could identify the type of CpG-DNA molecules and then mediate host immune responses to bacterial cells. Experiments have confirmed that intestinal epithelial cells can either be in a "tolerated" or "non-tolerated" state in the intestinal flora depending on the signal transduction pathway which TLRs mediate ^(10,11).

Some RNAs secreted by *Lactobacillus* are rRNA and tRNA

Lactobacilli are important members of the human intestinal

flora. They are a kind of probiotics which are widely used in food and in the treatment of infectious diseases. *Lactobacilli* play an important role in the preventing infection via their metabolites such as volatile fatty acids, lactolin and unknown proteins they secrete. No other metabolic substance with this effect has been found according to our knowledge. Our research team found 39 RNA sequences in *lactobacilli* medium filtrate at exponential growth phase, including two certain *lactobacillus*' rRNA and tRNA sequences. Theoretically, the bacteria would not die in the exponential growth phase and no DNA was found in the filtrate. The phenomenon of RNA secretion was reported by DEMAIN AL in *Bacillus subtilis* in 1965¹⁸⁾ but have not been found in *lactobacillus*, so RNAs in the exponential growth phase culture filtrate maybe secreted by *lactobacillus*. rRNA which comprise 80% of total RNAs in the cell, play an important role in the process of protein synthesis. We have observed that non-protein-coding micro-RNAs regulate genes to resist virus in the cell recently¹²⁾, however, we know little about the role RNAs secreted by prokaryotic cells. Intestinal flora interacted with the body as an ecosystem or acting on the body through the cell itself or by metabolites such as secreted proteins. As a kind of biological macromolecules, rRNA and tRNA molecules have similar structure with DNA perhaps they have similar extracellular biological activity. Researches on extracellular RNA and DNA's role in cells are significant to describe the relationship between the intestinal flora and the body. Our research showed that as biological molecules, RNAs contact with the intestinal epithelial cells more easily and even could reach the lower intestinal epithelial cells. This may be it may the molecular mechanism behind the influence of intestinal flora in the stability of the immune system of the body.

The immune activity of CpG-DNA

Recent studies showed that CpG-ODN has a certain immune activity on the body. CpG-DNA molecules can induce the activation of several signal molecules by associating with TLR9 and then activating NF- κ B at the same time blocking the activation of I κ B^{13, 14)}. In 1995, Krieg found that bacterial DNA can directly stimulate the proliferation of B cells and induce the secretion of IgM. They characterized by the structure of non-methylated cytosine guanine dinucleotide (CpG). This sequence appears in the genome of bacteria and viruses 20 times more than that of vertebrates¹⁵⁾. The binding of CpG-ODN and TLR9 induced the dimerization of TLR9 involved in signal transduction with the recruitment of myeloid differentiation factor 88 (MyD88). MyD88 plays a role by attracting IL-1 receptor associated kinase (IRAK) in inducing the degradation of I κ B and activating NF- κ B to regulate the synthesis and release of cytokines¹⁶⁾.

RNAs secreted by *Lactobacillus* can activate TLR9 and NF- κ B

TLRs are evolutionarily conserved pattern recognition

receptors expressed on the surface of a variety of immune cells. Intestinal epithelial cells recognize pathogens and other antigens to induce epithelial cells to produce cytokines, chemotactic factors, and anti-microbial peptides via TLRs. TLR9 exists in the basolateral and apical portion of the intestinal epithelial basement membrane in which TLR9 signaling pathways of IEC basolateral can activate NF- κ B¹⁷⁾. We studied the expression of TLR9 genes by RT-PCR and the experiments showed that RNAs secreted by *lactobacillus* can activate TLR9 compared to the control group. RNA increased by 180% while NF- κ B increased by 110%. The activity of CpG-ODN increased more significantly than RNAs that reached as high as 1719% and 439%. RNAs in vitro culture filtrate at logarithmic growth phase of *Lactobacillus* activated TLR9 receptor to increase the activity of NF- κ B suggesting that RNAs components secreted by *Lactobacillus* may be a new TLR9 ligand.

RNAs secreted by *Lactobacillus* can activate the secretion of IL-6 by HT-29 cells

IL-6 is a cellular immune factor which can be expressed in a variety of body cells and is regulated by TLRs via NF- κ B pathway which plays an important role in immunity¹⁸⁾. Our research detected IL-6 levels in the supernatant of RNAs group in HT-29 cells by ELISA. The results showed that nucleic acids secreted by *lactobacillus* could up-regulate IL-6 levels on HT-29 cells increasing by 28% compared to the control group, while the CpG-ODN group increased by 50%. RNAs can promote the expression of IL-6 *in vitro*. Taken together, our data indicate that nucleic acids secreted by *lactobacillus* could promote the levels of IL-6 *in vitro* by activating TLR9 to generate NF- κ B and that RNAs have similar immune activity with CpG-ODN.

Conclusion

The nucleic acids in *lactobacillus* DM8909 culture filtrate are RNAs which could increase IL-6 expression through the activation NF- κ B via LTR9 mediation *in vitro*. It has a similar immune activity with the CpG-ODN.

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