Int J Clin Exp Med 2015;8(2):2429-2434
www.ijcem.com /ISSN:1940-5901/IJCEM0003462

Original Article

Role of *CD14* and *TLR4* in type I, type III collagen expression, synthesis and secretion in LPS-induced normal human skin fibroblasts

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Received October 29, 2014; Accepted January 21, 2015; Epub February 15, 2015; Published February 28, 2015

Abstract: Objectives: The primary aim of this study was to investigate the role of CD14 and TLR4 in type I, type III collagen expression, synthesis and secretion in LPS-induced normal human skin fibroblasts. The secondary aim was to provide theoretical basis for the molecular mechanisms of scar formation induced by LPS. Methods: The normal skin fibroblasts cultured in vitro were randomly divided into four groups: 0.1 μg/mL LPS reference group, *CD14* pretreatment + LPS, *TLR4* pretreatment + LPS, *CD14* and *TLR4* pretreatment + LPS. The collagen DNA synthesis was assessed by ^{3}H-proline incorporation method. Real-time Quantitative PCR was used to detect type I, type III collagen mRNA expression. Results: Similar results were revealed for mRNA expression levels. The immunofluorescence staining suggested that type I and type III collagen were expressed in all investigated groups and that the expression was differentially downregulated in groups B, C, D. ELISA demonstrated markedly decreased levels in secreting type I, type III collagens and hydroxyproline in groups B, C, D ($P<0.05$), and the lowest level was detected in group D ($P<0.01$). Conclusion: Pretreatment with *CD14* or *TLR4* alone or their combination can significantly reduce the levels of type I and type III collagen expression, synthesis and secretion, with the most notable reduction detected in case of *CD14* and *TLR4* combined. We could thus conclude that both *CD14* and *TLR4* are involved in type I and type III collagen expression, synthesis and secretion in LPS-induced skin fibroblasts.

Keywords: Toll-like receptor 4, *CD14*, lipopolysaccharide, fibroblasts, collagen

Introduction

Hypertrophic scar histologically characterized by fibroblasts hyperplasia and excessive extracellular matrix accumulation is a result of the changes in some biological characteristics of fibroblasts [1, 2], and it is the gram negative bacteria and the endotoxin they contained, namely the lipopolysaccharide (LPS), are major causes of these changes [3, 4]. We have previously reported that LPS significantly promotes normal human skin fibroblasts in proliferation, synthesizing and secreting type I, III collagens; we also demonstrated that alternations in these biological characteristics are consistent with the biological behaviors of fibroblasts of hypertrophic scar tissues [3, 5, 6]. As yet, the mechanisms that underlie scar hyperplasia induced by severe trauma remain unclear. On the basis of our previous findings, we herein attempted to explore the molecular mechanisms by which LPS-induced normal human skin fibroblasts synthesizes and secretes collagens.

Toll like receptor 4 (*TLR4*) is expressed in all types of cell lines [7]. LPS combines with serum factor LPS binding protein (LBP) to form compounds, binds with membranous *CD14* on the surface of monocyte and macrophage, and interacts with *TLR4* to trigger downstream signaling pathways [8]. Despite the deficiency of membranous *CD14* on fibroblasts, soluble *CD14* could be used to activate the *TLR4* pathway, as previously reported in multiple studies [9, 10]. In the present work, we used *CD14* and *TLR4* in separation or in combination to treat normal human skin fibroblasts which were subsequently stimulated by LPS in an effort to observe the changes in collagen expression, synthesis and secretion, explore the role of *CD14* and *TLR4* in collagen expression, synthe-

sis and secretion in LPS-stimulated skin fibroblasts, provide theoretical basis for the molecular mechanisms of scar formation induced by LPS.

Materials and methods

Reagents and instruments

Reagents included RPMI 1640 basic medium, FBS, trypsin-EDTA (Gibco company, USA), green chain double antigen (PAA company, USA), normal human skin fibroblast cell lines (provided by Burn Research Institute at First Hospital Affiliated to General Hospital of PLA), 2,5-Diphenyloxazole (PPO), 1,4-Bis (5-phenyl-2-oxazolyl) benzene (POPOP), LPS (Sigma, 3H-USA), 3H-proline (China Institute of Atomic Energy), Trizol reagent box, type I, type III collagens and hydroxyproline ELISA Kit (Invitrogen company, American), M-MLV reverse transcription Kit (Takara Co., Japan), Real-time PCR amplification kit (Beijing Zhongyuan Co., China), and other chemical reagents, all made in China.

Instruments included CO_2 incubator (SANYO company, Japan), inverted fluorescence microscope (Leica company, Germany), voltage and current stabilizing electrophoresis, Bio-Rad 450 microplate reader system (Bio-Rad company, America), Tri-carb 2300TR liquid scintillation counter (Packard company, America), flow cytometry (Becton Dickinson, USA), and Real-Time PCR instrument (ABI 7500, America).

Grouping

The frozen normal human skin fibroblasts cell lines were first resuscitated, and then cultured in RPMI 1640 medium containing 10% FBS at 37°C in a 5% CO_2 atmosphere. 0.25% trypsin was used for cell digestion and passage. The third to the tenth generation cells were selected for experiments and divided into four groups: 0.1 g/mL LPS reference group (group A), *CD14* pretreatment + LPS group (group B), *TLR4* pretreatment + LPS group (group C), *CD14* combined with *TLR4* pretreatment + LPS group (group D).

End points

DNA synthesis of collagens

Cells at the concentration of 1.5×10^4/mL were inoculated in 96-well culture plate, with each well containing 0.2 mL cells and each group composed of 8 wells. After 24 h, all cells were cultured with 0.2 mL pretreatment liquid for 6 h, and then with 0.1 μg/mL LPS for 48 h. Subsequently, cells were cultured for another 6 h in the presence of 0.1 μg/mL LPS and 3H-proline (1 μCi/well). Then, the cells were conventionally digested, collected on the glass fiber filter paper, and sequentially washed with Duchenne phosphate buffer solution (D-PBS), 5% three chloro acetic acid and ethanol 3 times. After drying the glass fiber filter paper, the spotted paper was cut off and placed in the scintillation fluid. Counts per minute (CPM) in each hole were done using liquid scintillation counter.

Type I, III collagen mRNA expression

Cells at the concentration of 5×10^4/mL were inoculated in 6-well culture plate, with each well containing 3 mL cells and each group consisting of 4 wells. 24 h later, all cells were cultured with 3 mL pretreatment liquid for 6 h, followed by 0.1 g/mL LPS for 48 h. mRNA extraction was undertaken using 1 ml Trizol. Nucleic acid ultraviolet spectrophotometric was used to determine the purity and the concentration of RNAs. The RNAs in each group were reversely transcripted into cDNA according to the instructions of M-MLV kit, followed by Real-Time PCR amplification complying with the manufacture's recommendations. Primers for type I collagen (388bp) were 5'-AGACTGGCAACCTCAAGAAG-3' and 5'-TTCGGTTGGTCAAAGATAAA-3'. The reaction conditions were: 32 cycles of 95°C for 5 min, 94°C for 30 s, annealing at 60°C for 1 min, followed by 72°C for 10 min. Primers for type III collagen (120bp) were 5'-ATGGTTGCACGAAACACACT-3', and 5'-CTTGATCAGGACCACCAATG-3'. The reaction conditions were: 40 cycles of 95°C for 15 min, 94°C for 8 s, annealing at 60°C for 34 s, followed by 72°C for 10 min. Primers for Beta-actin (143bp) included 5'-GGGTGTGAACCATGAGAAGT-3', and 5'-GGCATGGACTGTGGTCATGA-3'.

Type I, type III collagen expression

Normal human skin fibroblasts were inoculated in 24-well plate with coverslips at the concentration of 5×10^4/mL. Each well contained 1 mL cells and each group had 4 wells. After incubation for 24 h, all cells were incubated in 1 mL pretreatment liquid for 6 h, followed by 0.1 g/mL LPS for another 48 h. The coverslips were removed out and fixed by 4% paraformaldehyde

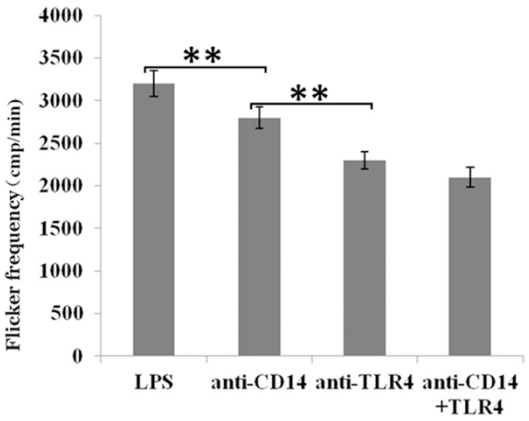

Figure 1. The levels of collagen DNA synthesis of human normal skin fibroblasts in different groups.

for 40 min, followed by paraformaldehyde removal, PBS washing (3 times, each washing 5 min), addition of 0.3% Triton transparent film for 30 min, PBS washing, normal goat serum closed for 30 min, and washing with PBS 3 times. 100 L rabbit anti human I type, type III collagen monoclonal antibodies were dropped on each coverslip and incubated at 4°C overnight. After washing 3 times with PBS, fluorescein isothiocyanate (FITC) was added to label goat anti rabbit second antibody, with the final step of lightless reaction at 37°C for 30 min. The inverted fluorescence microscope was used to take photos.

Type I, III collagen and hydroxyproline secretion

The cells were treated in the same way as described in 1. 3. 1. 8 holes were prepared for each group. Cell counting was conducted after 0.4% trypan blue staining. The secretion of type I, III collagens and hydroxyproline present in supernatant was detected by ELISA. The content of type I, III collagen and hydroxyproline was determined based on the standard curve drawn according to the detected OD450 values, and converted into the secretion of 1 × 106 cells.

Statistical analysis

Experiments were carried out in a minimum of three times. Statistical analyses were done using SPSS16.0 software. Data were expressed as mean ± standard deviation. Between-group difference was assessed with one-way analysis of variance (ANOVA). Pairwise comparisons were carried out using Student-Newman-Keuls-q test (SNK-q). Kruskal-Wallis H test was performed in case of heterogeneity of variance. The significance threshold was fixed at $P<0.05$.

Results

Collagen DNA synthesis

The collagen DNA synthesis in normal skin fibroblasts was detected by [3H]-proline incorporation method. The results showed that the synthesis levels were significantly reduced in groups B, C, D compared with group A, with group D showing the lowest level ($P<0.01$) (see **Figure 1**).

mRNA expression of type I, type III collagens

Real-time Quantitative PCR showed a significant reduction in mRNA expression of type I, type III collagens in groups B, C, D as compared to group A ($P<0.05$). The lowest level was detected in group D ($P<0.01$) (see **Figure 2**).

Intracellular expression of type I, type III collagens

The obtained results of immunofluorescence staining revealed that type I, type III collagens were expressed in all groups being investigated. The results also revealed different degrees of reduction in groups B, C, D as compared to group A (see **Figure 3**).

Secretion of type I, type III collagens and hydroxyproline

Secretion of type I, type III collagens and hydroxyproline in normal human skin fibroblasts was detected using the ELISA assay. We found that the content was significantly less in groups B, C, D than in group A ($P<0.05$), with the group D showing the lowest levels ($P<0.01$) (see **Table 1**).

Discussion

Fibroblasts are the known effector cells of hyperplastic scar which usually appears after deep burns and severe infections. Results of our previous research showed that LPS significantly enhances normal human skin fibroblasts in collagen expression, synthesizing and secreting collagens; it also substantially reduces the amount of collagenase, and thereby reduces the degradation of collagen fibers; the alternations in biological characteristics are in line with the behaviors of hypertrophic scar tissues [5]. Previous work has provided evidence that

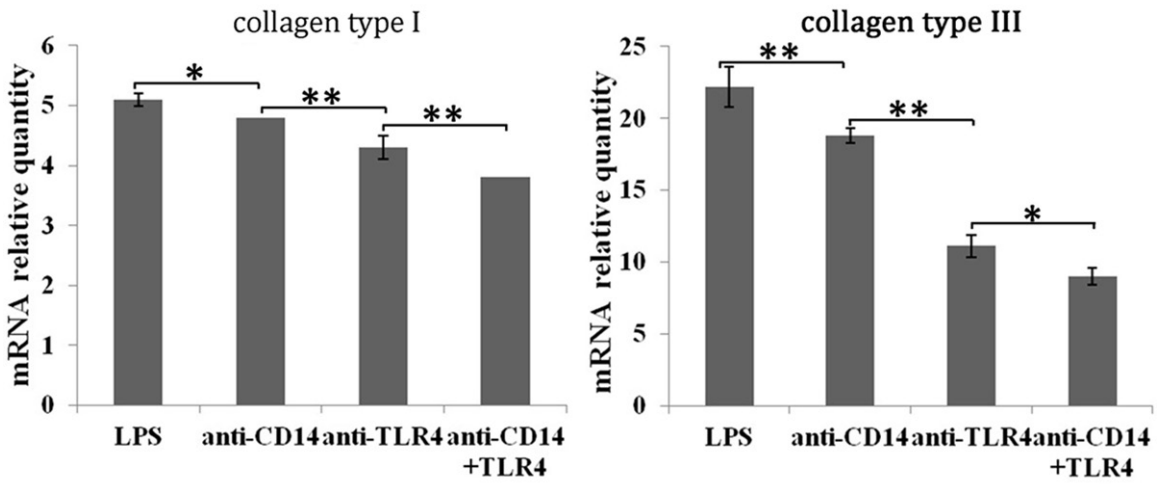

Figure 2. The levels of collagen type I and type III mRNA of human normal skin fibroblasts in different groups.

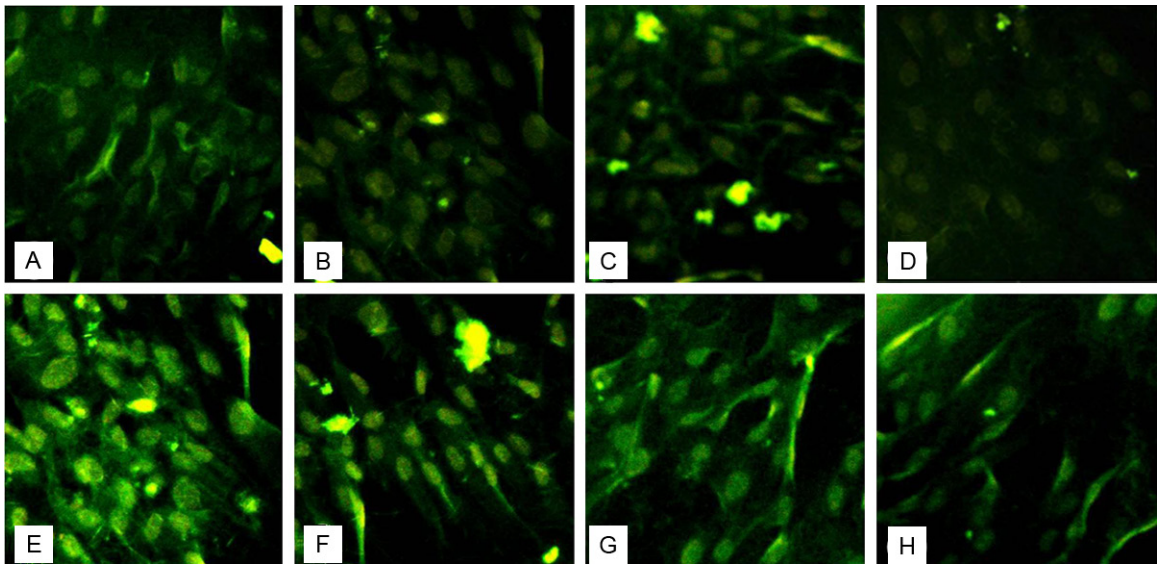

Figure 3. The expression levels of collagen type I and type III of human normal skin fibroblasts in different groups. A: Collagen type I in group A; B: Collagen type I in group B; C: Collagen type I in group C; D: Collagen type I in group D; E: Collagen type III in group A; F: Collagen type III in group B; G: Collagen type III in group C; H: Collagen type III in group D.

LPS significantly promotes proliferation of lung fibroblasts through the *TLR4* signaling mechanism [11]. In addition, LPS activates various proteins within fibroblasts, including protein tyrosine kinase, monocyte chemoattractant protein-1 (MCP-1), nuclear transcription factor (NF-kappaB), activated protein-1 (AP-1), IL-1 receptor associated kinase (IRAK) and extracellular signal regulated kinase 1, protein kinase 2. The aforementioned activations are clearly linked with cell migration, proliferation and inflammatory responses [12-17].

We have previously demonstrated that the expression, synthesis and secretion of type I, type III collagen of normal human skin fibroblasts stimulated by 0.1 g/mL LPS are similar to those of hypertrophic scar fibroblasts derived from the same individual [5]. Therefore, we selected 0.1 g/mL LPS to culture normal human skin fibroblasts. The results of this study suggested that the DNA synthesis levels, type I, type III collagen mRNA expression, as well as type I, type III collagen and hydroxyproline secretion were remarkably reduced after pre-

Table 1. The levels of collagen type I, collagen type III and hydroxyproline secretion of human normal skin fibroblasts in different groups

group	*quantity (n)*	*collagen type I*	*ollagen type III*	*hydroxyproline*
A	8	285.37±31.64	590.41±21.24	803.31±26.34
B	8	235.42±20.91*	532.54±23.95*	695.27±19.23**
C	8	155.37±11.18**	429.08±38.01**	463.82±13.19**
D	8	128.06±18.55**	389.90±18.94**	415.04±23.79**

Compared with group A, *P<0.05, **P<0.01.

treatment with *CD14* and *TLR4* either in isolation or in combination, with the lowest levels detected in the skin fibroblasts pretreated with *CD14* and *TLR4* combined. These data seem to suggest that both *CD14* and *TLR4* are involved in type I, type III collagen expression, synthesis and secretion in LPS-stimulated human normal skin fibroblasts.

As proline is an important amino acid of the molecule collagen peptide with stable content and the content is especially low in other tissues, we selected 3H-proline incorporation assay to detect the collagen DNA synthesis. Hydroxyproline is a major component of the structural macromolecules collagen peptide. Therefore, the content of hydroxyproline can well reflect the skin collagen synthesis [18]. Results of the present work have shown that both use of *CD14* and *TLR4* in separation and use of *CD14* and *TLR4* in combination result in significant reductions in collagen DNA and collagen synthesis, with the sharpest reduction ascribed to the latter pretreatment.

In summary, the obtained results suggest that both *CD14* and *TLR4* are involved in the collagen expression, synthesis and secretion in LPS-induced normal human skin fibroblasts. Our findings may shed light on the signaling mechanisms underlying scar formation induced by LPS.

Acknowledgements

This work was supported by the National Natural Science Foundation of China (8137-2053).

Disclosure of conflict of interest

None.

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