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Original Article

Association of *CD14* and *TLR4* with LPS-stimulated human normal skin fibroblasts in immunophenotype changes and secretion of TGF-β1 and IFN-γ

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Abstract: Objectives: We attempted to explore the association of *CD14* and *TLR4* with LPS-stimulated human normal skin fibroblasts in immunophenotype changes and secretion of TGF-β1 and IFN-γ, and to expand the current knowledge of the mechanisms that underlie LPS-induced scar formation. Methods: We randomized the human normal skin fibroblasts cultured in vitro into four groups. The expression profile of immune phenotypes was determined by immunohistochemical staining. Ultrastructure of cells was observed by use of transmission electron microscopy. Secretion status of TGF-β1 and IFN-γ was inspected using ELISA assay. Results: Compared with group A, the expressions of α-SMA and α1 (I) procollagen in groups B, C, D were lower, and it in group D were the lowest in all groups. The cells in group A were diversification under the electron microscope, and the ratio of the nuclear to plasma of the fibroblasts was large, with unregular nuclear membrane, more Golgi apparatus, rough endoplasmic reticulum, and microfilament and canaliculus appeared. The ultrastructure of the fibroblasts in group B, C, D was spindle and the nuclear was large, with regular nuclear membrane, more Golgi apparatus, rough endoplasmic reticulum. ELISA assay indicated that the secretion of TGF-β1 markedly lowered in groups B, C, D in comparison to group A, with the most marked decline observed in group D. Interestingly, we found significantly increased IFN-γ secretion in groups B, C, D ($P < 0.05$), with the latter group showing the most notable increase ($P < 0.01$). Conclusion: These data suggest that both combined and isolated use of *CD14* and *TLR4* significantly reduce α-SMA expression levels, the number of α1 (I) pro-collagen positive cells, and TGF-β secretion, while substantially increased IFN-γ secretion. The reduction and increase are especially notable when pretreating with *CD14* and *TLR4* combined. Here we thus draw a conclusion that both *CD14* and *TLR4* are associated with the immunophenotype changes and secretion of TGF-β1 and IFN-γ in LPS-stimulated human normal skin fibroblasts.

Keywords: Lipopolysaccharide, fibroblast, collagen, toll like receptor 4, *CD14*

Introduction

Hypertrophic scar stems as a result of hyperproliferative lesions formed during the healing of severe trauma of skin tissues [1-3]. While existing studies have shown that gram negative bacterial lipopolysaccharide (LPS) and skin fibroblasts are major components in the formation of hyperplastic scar [4, 5], limited knowledge remains concerning the pathogenesis. Data documented in our previous work of human normal skin fibroblasts indicated that 0.1 μg/mL LPS substantially increases the expression of proliferating cell nuclear antigen (PCNA), alpha smooth muscle actin (α-SMA), alpha 1 (I) pro-collagen (α1 (I) pro-collagen), and the secretion of transforming growth factor beta 1 (TGF-β1), whereas significantly inhibited interferon-gamma (IFN-γ) secretion. In addition, the alternations of these biological characteristics are almost identical with those exhibited in hypertrophic scar fibroblasts [6-8].

Toll like receptors (TLRs) are fundamental pattern recognition receptors with a significant involvement in protecting hosts from a variety of microorganisms. Kim et al. have demonstrated strong evidence that LPS activates the TGF-β signaling pathway in conjunction with *TLR4*, thereby promoting liver fibrosis [9]. Nonetheless, it is indecisive over whether or not *CD14* is involved in LPS and *TLR4* interaction activated downstream signals [10, 11]. We hypothesize that the involvement of *CD14* may be deter-

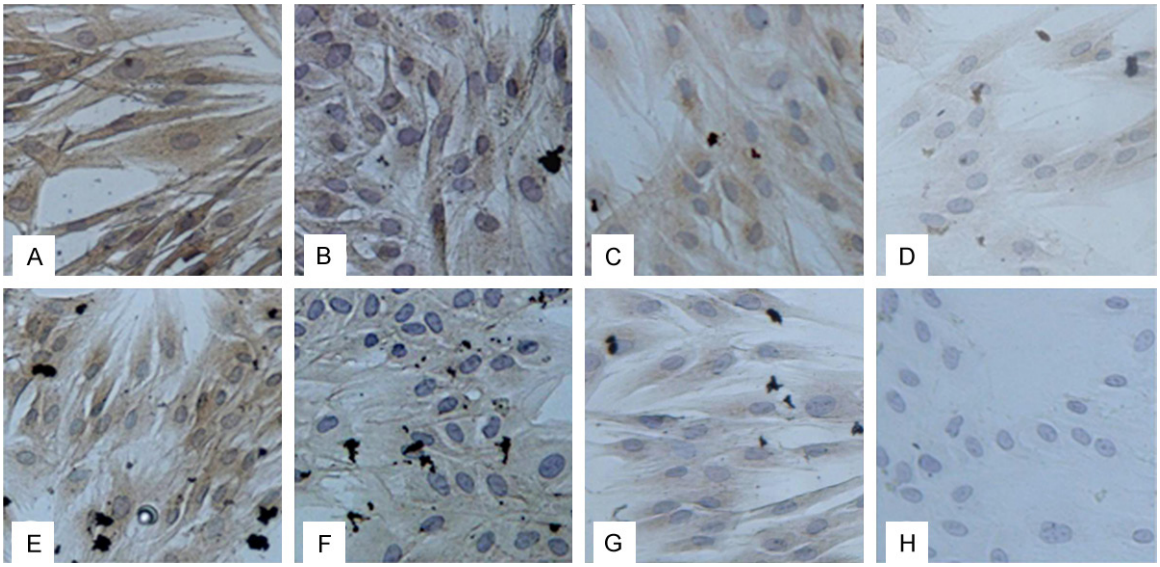

Figure 1. Immunophenotyping of human normal skin fibroblasts in different groups (100 ×). (A-D) Corresponded to α-SMA expression in groups (A-D), respectively; (E-H) Represented α1 (I) pro-collagen expression in groups (A-D), respectively.

mined by the signaling pathway LPS and *TLR4* initiate. To clarify this issue, we used *TLR4* and *CD14* in combination and in isolation, in an attempt to observe their effects on the immunophenotype changes and secretion of TGF-β1 and IFN-γ in LPS-stimulated human normal skin fibroblasts. We also aimed to provide deeper insights into the mechanism of LPS-induced scar formation.

Materials and methods

Reagents and instruments

The reagents selected for this study included RPMI 1640, FBS, trypsin-EDTA (Gibco, America), antibiotics of penicillin and streptomycin (PAA, America), normal skin burn patient fibroblast cell lines (offered by Burn Research Institute), LPS (E. coli055:B5) (Sigma, America), primary antibody rabbit anti human α-SMA and α1 (I) pro-collagen (Beijing Boisynthesis, China), TGF-β1, IFN-γ, and ELISA Kit (R&D, America). Major instruments were CO2 incubator (SANYO, Japan), upright microscope (Olympus, Japan), microplate reader model (Bio-Rad, America), and transmission electron microscope (Hitachi, Japan).

Grouping

The frozen cell lines were first resuscitated, and subsequently cultured in RPMI 1640 medium containing 10% FBS at 37°C with 5% CO_2. Cell digestion and passage were done with 0.25% trypsin. The third to the tenth generation cells were selected for later experiments and randomized into four groups (group A = 0.1 g/mL LPS group, group B = *CD14* pretreatment + LPS, group C = *TLR4* pretreatment + LPS, group D = *CD14* combined with *TLR4* pretreatment + LPS).

Changes in phenotypes

Normal human skin fibroblasts (5×10^4/mL) were cultured for 24 h in prepared coverslips in 24-well plates (1 mL/well). The 4 wells were prepared for each group being investigated. Then the cells were cultured with pretreatment liquid (1 mL) for 6 h and with 0.1 μg/mL LPS for another 48 h. The coverslips were removed and subject to immunohistochemical staining. α-SMA (100 μl) and α1 (I) pro-collagen (100 μl) were dripped on each coverslip, followed by DAB staining according to the manufacture's instructions.

Ultrastructure

Fibroblasts were cultivated in T-25 flasks containing RPMI 1640 medium for 24 h, followed by cultivation with pretreatment liquid (5 mL) for 6 h and 0.1 μg/mL LPS for 48 h. After digestion with 0.25% trypsin, the cells totaling about 1,000,000~10,000,000 were transferred into

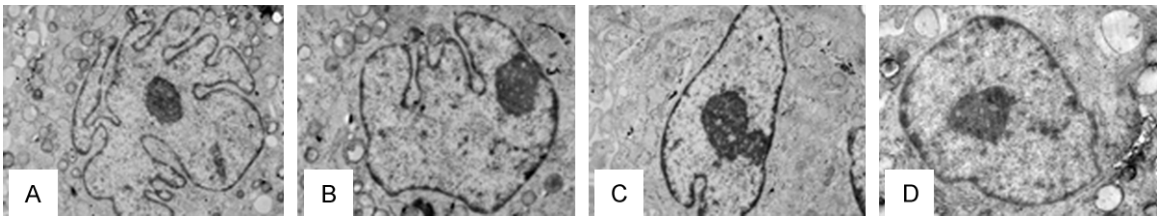

Figure 2. Ultrastructure of human normal skin fibroblasts in different groups (TEM, 10000 ×). (A-D) Corresponded to groups (A-D), respectively.

Table 1. Levels of TGF-β1 secreted by human normal skin fibroblasts ($\bar{x}$ ± s, n = 6, pg/ml)

Groups	*n*	*12 h*	*24 h*	*48 h*
Group A	8	306.02 ± 6.35	517.62 ± 11.77	933.32 ± 11.29
Group B	8	280.32 ± 8.12**	480.73 ± 8.03**	850.17 ± 13.91**
Group C	8	240.93 ± 10.23**	430.21 ± 13.91**	720.05 ± 20.21**
Group D	8	208.17 ± 12.14**	378.35 ± 15.78**	623.35 ± 25.37**

Group A as the reference group, *P < 0.05, **P < 0.01.

Table 2. The levels of IFN-γ secreted by human normal skin fibroblasts ($\bar{x}$ ± s, n = 6, pg/ml)

Groups	*n*	*12 h*	*24 h*	*48 h*
Group A	8	50.53 ± 4.78	92.57 ± 3.28	199.31 ± 18.67
Group B	8	58.87 ± 6.21	101.39 ± 7.21	248.84 ± 20.25**
Group C	8	76.29 ± 3.21**	132.07 ± 5.27**	331.17 ± 13.55**
Group D	8	88.32 ± 4.37**	156.70 ± 6.83**	382.32 ± 10.74**

Group A as the reference group, *P < 0.05, **P < 0.01.

centrifuge tubes (10 mL), then fixed with precooled 2% glutaraldehyde (4°C) and 1% osmium acid, followed by dehydration with acetone, embedding with EPON812, sections, and staining with uranyl acetate and citric acid aluminum. Transmission electron microscope (JEM-200 EX) was utilized to take photos and make records.

TGF-β1 and IFN-γ secretion

Cells (1.5 × 10^4/mL) were inoculated in 96-well plates (0.2 mL/well, 8 wells prepared for each group) for 24 h, then with pretreatment liquid (0.2 mL) for 6 h and with 0.1 ug/mL LPS for 12 h, 24 h and 48 h respectively for later use. The cells were counted after stained with 0.4% trypan blue. The secretion of TGF-β1 and IFN-γ contained in the supernatant was detected by ELISA method. Standard curves were made according to the observed OD450 values, to measure the content of TGF-β1 and IFN-γ.

Statistical analyses

Data were expressed as mean ± SD. One-way analysis of variance (ANOVA) was used to compare the difference between groups. Pairwise comparisons were done using SNK-q test. In case of heterogeneity of variance, between-group comparisons were done with Kruskal-Wallis H test and Nemenyi test was performed for pairwise comparisons. All statistical tests were carried out using SPSS software for Windows (ver. 16.0, SPSS, Inc., Chicago, IL, USA). A P value < 0.05 was considered statistically significant.

Results

Phenotype changes

We observed brownish yellow or brown cytoplasms after stained with α-SMA and α1 (I) procollagen. Compared to group A, groups B, C, D showed distinct degrees of reduction in the number of positive cells and positive expression, with the most notable reduction observed in group D (**Figure 1**).

Ultrastructural changes

Using transmission electron microscope, we found diverse morphologies, high ratio of nucleoplasm, irregular nuclear membrane, and a large number of rough endoplasmic reticulums, Golgi complexes, microtubules and microfilaments in endochylema in group A. The ultrastructure observed in group B was similar to group A, but the nuclear membrane was more regular. In groups C and D, the cells were spindle-shaped with large oval nuclei and notable nucleolus. In

addition, there were numerous rough endoplasmic reticulums, Golgi complexes and tiny mitochondrium present in endochylema (**Figure 2**).

TGF-β1 and IFN-γ secretion

The content of TGF-β1 contained in the supernatant at 12 h, 24 h, and 48 h was detected by ELISA. A significantly reduced content was observed in groups B, C, D, with the latter group showing the most significant reduction. Interestingly, the content of IFN-γ remarkably increased in groups B, C, D compared with group A ($P < 0.05$) and the most remarkable increase was observed in group D ($P < 0.01$), as shown in **Tables 1** and **2**.

Discussion

Hyperplastic scar arises as a consequence of the changes in biological characteristics of normal skin fibroblasts, with the main histological features including fibroblast hyperplasia and excessive accumulation of extracellular collagen matrix [12]. The mechanisms underlying the progression of hyperplastic scar remain incompletely understood. We have previously reported that LPS not only has a central role in significantly promoting the proliferation of normal human skin fibroblasts, also substantially enhances collagen synthesis and secretion [6, 7]. Li et al. and Yang et al. lend further support for the fundamental role of LPS in immunophenotype changes, secretion of some cytokines essential for promoting cell proliferation and differentiation in normal human fibroblasts [13, 14]. Recent data demonstrated that activated fibroblasts produce TGF-β1 and IFN-γ through autocrine or paracrine, and the two cytokines are clearly associated with the formation of hyperplastic scar [15]. TGF-β1 significantly promotes fibroblast differentiation and proliferation as well as extracellular matrix deposition, while IFN-γ inhibits the proliferation of fibroblast, facilitates apoptosis, downregulation of type I, type III collagen gene expression, and triggers the production of collagenase gene, thereby preventing the appearance of extracellular matrix [16-19]. In addition, IFN-γ suppresses fibroblast differentiation and use of anti-TGF-β1 contributes to the inhibition of liver fibrosis, as corroborated in an experimental study conducted by Luo et al. [20].

Here we have demonstrated that both separate and combined use of *CD14* and *TLR4* monoclonal antibodies significantly reduce the expression of α-SMA, the number of α1 (I) pro-collagen positive cells and secretion of TGF-β1, with the most notable reduction observed when cells were pretreated with *CD14* and *TLR4* combined. We also noted obviously weaker effects of LPS in inhibiting IFN-γ secretion, again with the combination of *CD14* and *TLR4* resulting in the most significant inhibitory impact.

To sum up, we demonstrate here that both *CD14* and *TLR4* are associated with LPS-stimulated normal human fibroblasts in immunophenotype changes and TGF-β1 and IFN-γ secretion. These observations may shed light on the signaling mechanism of LPS-induced scar formation. Additional studies are required to confirm or refute the findings.

Disclosure of conflict of interest

None.

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