Cytokine 102 (2018) 1–6

Contents lists available at ScienceDirect

Cytokine

journal homepage: www.elsevier.com/locate/cytokine

Polyriboinosinic-polyribocytidylic acid facilitates interleukin-6, and interleukin-8 secretion in human dermal fibroblasts via the JAK/STAT3 and p38 MAPK signal transduction pathways

Zhang Ying Zhu[a], Cong Zhuo Jia[c], Jian Min Luo[a,*], Li Wang[b,c,d,*]

[a] Department of Pathophysiology, Shantou University Medical College, 5150412, People's Republic of China
[b] Shenzhen University General Hospital, 518055, People's Republic of China
[c] Department of Dermatology, First Affiliated Hospital, Shantou University Medical College, 515041, People's Republic of China
[d] Huizhou Municipal Hospital, People's Republic of China

ARTICLE INFO

Keywords:
Human dermal fibroblasts
PolyI:C
Interleukin-6 (IL-6)
Interleukin-8 (IL-8)
Wound healing
Cytokine
STAT3, p38 MAPK

ABSTRACT

Polyriboinosinic-polyribocytidylic acid (polyI:C) is a viral dsRNA analoguethat promotes wounds healing, accelerates re-epithelialization, granulation and neovascularization, and induces pro-inflammatory cytokine release. Little is known about polyI:C mediated induction of inflammatory mediators in human dermal fibroblast (HDFs), which form the primary scaffold for epithelial cells covering the wound. Here, we found that polyI:C enhances IL-6 and IL-8 mRNA expression and induces of IL-6 and IL-8 production in a concentration-dependent and time-dependent manner in HDFs. PolyI:C treatment rapidly increased phosphorylation level of both STAT3 and p38 mitogen-activated protein kinase (MAPK). Moreover, pretreatment with AG490, a Janus kinase (JAK) inhibitor, inhibited polyI:C-induced STAT3 phosphorylation and subsequent IL-6 and IL-8 release. Conversely, pretreatment with SB203580, a selective inhibitor of p38 MAPK, blocked p38 MAPK phosphorylation and IL-6 and IL-8 expression. In conclusion, polyI:C induces IL-6 and IL-8 production in HDFs via the JAK/STAT3 and p38 MAPK signaling pathways.

1. Introduction

Polyriboinosinic-polyribocytidylic acid (polyI:C) is a viral dsRNA analogue and promotes wound healing *in vivo* [1]. Toll-like receptors (TLRs) are pattern-recognition receptors that mediate innate immune responses and the host defense against pathogens [2]. TLRs have an important role in tissue homeostasis during the inflammatory response, based on regulating tissue repair and regeneration.

In double-stranded RNA (dsRNA)-related signaling cascade activation, TLR3 triggers skin regeneration [3]. Our previous study found that TLR3 and its ligand, polyI:C, facilitate human and murine skin repair [4]. Subsequent studies in mouse models have shown that polyI:C application to a wound boosts neutrophils and macrophages recruitments, leading to elevated macrophage inflammatory protein-2 (MIP-2/CXCL2) expression [4].

Cytokines, chemokines and growth factors also accelerate fibrogenesis by enhancing fibroblast proliferation and collagen synthesis and promoting granulation tissue remodeling [5,6]. Many types of cytokines are released upon TLR stimulation, which have important roles during wound healing: IL-6 and IL-8 are the most abundant cytokines in wound fluid by surgical drains [7].

Different lymphoid and non-lymphoid cells, including T and B lymphocytes, fibroblast, keratinocytes and some tumor cells, release IL-6 [8]. IL-6 has pleiotropic functions that predominantly regulate the immune system but also some various physiological processes in many organs [9]. Such as inducting acute-phase proteins and inflammation, antigen-specific immune responses, host defense mechanisms and hematopoiesis [10]. *In vitro* wound healing assays have also shown that IL-6 accelerates biliary-cell migration [11].

Monocyted-/macrophages, epithelial cells, fibroblasts and hepatic cells produce IL-8, which affects chemotaxis and proliferation of inflammatory cells [12] and regulates the function and recruitment keratinocytes and fibroblasts [13]. IL-8 is a potent facilitator of angiogenesis and promotes endothelial cell survival, proliferation and matrix metalloproteinases (MMP) deposition [14]. The level of IL-8 production by normal, healthy skin cells is markedly lower than that of an unhealed wound biopsy [15].

PolyI:C stimulates various cells to release different pro-/anti-

* Corresponding authors at: Department of Pathophysiology, Shantou University Medical College, 24 Xinling Road, Shantou 515041, People's Republic of China (J.M. Luo). Department of Dermatology, Shenzhen University General Hospital, Shenzhen 518055, Guangdong Province, People's Republic of China (L. Wang).
E-mail addresses: jml@stu.edu.cn (J.M. Luo), lwang12@stu.edu.cn (L. Wang).

https://doi.org/10.1016/j.cyto.2017.12.012
Received 8 September 2017; Received in revised form 7 December 2017; Accepted 9 December 2017

inflammatory cytokines, including IL-8 from human aneuploid immortal keratinocytes and human epidermal keratinocytes [16,17]. The A549 lung adenocarcinoma cell line is particularly responsive to polyI:C in terms of upregulating IL-6, IL-8, MCP-1, GRO and MCP-1 secretion. PolyI:C also upregulates IL-1α, IL-8, RANTES and GRO in NCI-H292 cell, but instead downregulates MCP-1. Finally, MMP10 and MMP13 are released by the A549 and NCI-H292 cells following polyI:C exposure [18]. Interestingly, NCI-H1299 cells are insensitive to polyI:C as these cells produce abundant basal levels of cytokines that cannot be further enhanced.

The differences in TLR biology and inflammatory mechanisms between humans- and murine models require further investigation. Here, we dissected the IL-6, IL-8 and intracellular signaling pathways in primary cultured HDFs activated by polyI:C.

2. Material and methods

2.1. Cell and reagents

Human dermal fibroblasts (HDFs) were obtained from four samples (healthy volunteers, two at age 18, one at 20, one at 21) of foreskins undergoing circumcision in First Affiliated Hospital, Shantou University Medical College. Subsequently procured from the Laboratory for Allergy and Inflammation Research. Cell were made as previously described [19]. This study adhered to the tenets of the Declaration of Helsinki, and all subjects signed an informed consent form before undergoing circumcision. HDFs were cultivated in RPMI medium 1640 (Gibco RRL/Life-technologies, Rockville, USA), supplemented with 10% (v/v) fetal bovine serum (Gibco RRL/Life technologies, Rockville, MD, USA), 100 U/ml penicillin and 100 μg/ml streptomycin (Solarbio) and incubated in sterile dishes at 37 °C in humidified atmosphere with 5% CO_2. PolyI:C was obtained from InvivoGen. BCA Protein Assay Kit was purchased from TIANGEN Biotech (Beijing), and a GoScript™ Reverse Transcription System was obtained from Promega. The QuantiFast SYBR Green PCR Master Mix was purchased from Qiagen. AG490 and SB203580 were purchased from Selleck. Phospho-STAT3 antibody and total-STAT3 antibody, anti-Phospho-p38 antibody and anti-p38 antibody, HRP-conjugated goat anti-mouse IgG, HRP-conjugated goat anti-rabbit IgG, anti-β-actin antibody were bought from Cell Signaling Technology (Beverly, MA, USA). ELISA kits to quantify human interleukin 6 and interleukin 8 ELISA kits were purchased from Cusabio. Other reagents, such as salt and buffer components, were analytical grade and were obtained from Sigma (St. Louis, MO, USA).

2.2. Challenge of HDFs with polyI:C

HDFs were plated in 6-well tissue culture plates (25×10^5 per well). When cells reached 85% confluence, growth media was replaced with serum-free medium, and cells were serum-starved overnight. After washing 3 times with PBS, cells were challenged with various concentrations of polyI:C (0, 0.5, 1, 5, 10, 20 μg/ml) in fresh serum-free medium.

2.3. Reverse transcription and quantitative real time PCR (qRT-PCR)

After exposure to various stimuli for 24 h, total RNA was extracted using TRIzol reagent (Life Technologies, USA) and cDNA was synthesized from 2 μg total RNA using a GoScript™ Reverse Transcription System according to the manufacturer's instructions. Human IL-6 and IL-8 mRNA levels were determined by qRT-PCR with specific IL-6 and IL-8 primers based on the IL-6 and IL-8 sequences reported in Genbank and designed using Primer Premier 5 software (Table 1). Actin was used as an internal control. The cycling conditions were as follows: denaturation at 95 °C for 1 min and amplification by cycling 35 times at 94 °C for 30 s, 60 °C for 20 s, and 72 °C for 30 s. Target gene expression was determined using the comparative cycle threshold (ΔΔCT) method and normalized to β-actin.

Table 1
Primer sequences and size for IL-6, IL-8 and β-actin.

Primer	Sequence	Size of product (bp)
IL-6		
Sence	5′-CTGGTCTTTTGGAGTTTGAGGTAT-3′	140
Antisence	5′-TGTGGTTGGGTCAGGGGTGGTTAT-3′	
IL-8		
Sence	5′-TCAAAAACTTCTCCACAACCCTC-3′	166
Antisence	5′-GAAGGTGACAGCAGTCGGTTGGA-3′	
β-actin		
Sence	5′-GAAGGTGACAGCAGTCGGTTGGA-3′	156
Anitsence	5′-GAGAAGTGGGGTGGCTTTTAGGA-3′	

2.4. IL-6 and IL-8 ELISA

IL-6 and IL-8 levels in culture supernatants were measured using the ELISA kits, according to the manufacturer's instructions. HDFs were stimulated with increasing concentrations of polyI:C (0, 0.5, 1, 5, 10, 20 μg/ml) in fresh serum-free medium for an incubation period of 0, 4, 8, 12, 18, 24 and 30 h. The culture supernatants were then collected for subsequent analysis and stored at −80 °C after centrifuged at 4 °C.

For experiments using inhibitors, the cells were pre-incubated with 40 μM AG490 or 20 μM SB203580 for 1 h followed by stimulation with polyI:C for an additional 24 h. The culture media was then collected as described and IL-6 and IL-8 levels were determined.

2.5. Western blotting

HDFs were stimulated with 20 μg/ml polyI:C in fresh serum-free medium for 30 min, 1, 2, 4 or 6 h. After three washes with PBS, the cells were lysed in RIPA lysis buffer supplemented with phenylmethanesulfonyl fluoride for 15 min at 4 °C. Cell debris was removed by 30-min centrifugation at 12,000*g*. Protein concentrations of the whole-cell lysates were determined using a BCA Protein Assay Kit, and equal amounts of proteins were separated by 12% SDS-PAGE before semi-dry transfer PVDF membranes, according to the manufacturer's instructions. Membranes were blocked with 5% skimmed milk in TBST (25 mM Tris, 137 mM NaCl, 2.7 mM KCl, 0.1% Tween 20, pH 7.6) at room temperature for 1 h, washed twice with TBST, and then incubated overnight at 4 °C with a primary antibody against phospho-p38, p38, phospho-STAT3 or STAT3, and β-actin (for normalization). After three washes with TBST, the membranes were incubated with HRP-conjugated goat anti-rabbit antibody or HRP-conjugated goat anti-mouse antibody for 1 h at room temperature. The protein bands with visualized by chemiluminescence, according to manufacturer's instructions. Radiographs were imaged on photographed with a digital scanning system and densitometry analysis of the immunoblots was carried out using Image J software. The relative levels of phospho-STAT3 and phospho-p38 were expressed following normalization to β-actin.

For studies using STAT3 or p38 MAPK inhibitors, HDFs were treated with 40 μM AG490 (STAT3 inhibitor) or 20 μM SB203580 (p38 MAPK inhibitor) for 30 min prior to adding 20 μg/ml polyI:C. The cells were harvested for western blot analysis as described above, after incubation for 30 min or 2 h.

2.6. Statistical analysis

All data are expressed as the means ± standard error (SE) for separate experiments. Statistical analyses were performed using SPSS 20.0. Differences were evaluated using analysis of variance (ANOVA) and least significant difference (LSD) *t*-test for multiple-comparison testing. A $P < 0.05$ was considered statistically significant and is