D-4F, an apolipoprotein A-I mimetic, suppresses IL-4 induced macrophage alternative activation and pro-fibrotic TGF-β1 expression

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ABSTRACT

Context: We reported that D-4F, an apolipoprotein A-I (Apo A-I) mimetic polypeptide with 18 α-amino acids, suppressed IL-4 induced macrophage alternative activation and TGF-β1 expression in phorbol 12-myristate 13-acetate (PMA) treated human acute mononuclear leukemia cells (THP-1).

Objective: Macrophage alternative activation, TGF-β1 and epithelial-mesenchymal transition (EMT) are intensively involved in pulmonary fibrosis. Recent studies demonstrated that Apo A-I resolved established pulmonary fibrotic nodules, and D-4F inhibited TGF-β1 induced EMT in alveolar cells. Therefore, this study evaluated the effects of D-4F on IL-4 induced macrophage alternative activation and TGF-β1 expression.

Materials and methods: THP-1 cells were simulated with PMA (100 ng/mL) for 48 h and treated with medium control, IL-4 (20 ng/mL) alone, or IL-4 (20 ng/mL) in the presence of D-4F (1, 5, and 10 μg/mL) for 24 and 48 h. Flow cytometry, RT-PCR and ELISA evaluations were performed to investigate the subsequent effects of D-4F.

Results: Compared to stimulation with IL-4 alone, 1, 5, and 10 μg/mL of D-4F reduced alternative activation by 45.38%, 59.98%, and 60.10%, increased TNF-α mRNA levels by 8%, 11%, and 16% and decreased TGF-β1 mRNA levels by 21%, 37%, and 39%, respectively (all p<0.05). In addition, TNF-α protein levels increased from 388 pg/mL (IL-4 alone) to 429, 475, and 487 pg/mL (1, 5, and 10 μg/mL D-4F), while TGF-β1 protein levels dropped from 27.01 pg/mL (IL-4 alone) to 19.15, 12.27, and 10.47 pg/mL (1, 5, and 10 μg/mL D-4F).

Conclusion: D-4F suppressed IL-4 induced macrophage alternative activation and pro-fibrotic TGF-β1 expression.

Introduction

Macrophages are classified into two major phenotypes based on physiological functions and activation mechanisms (Gordon 2003). The M1 phenotype undergoes classical activation through TNF-α (TNF-α) in host defence and anticancer activities. The M2 phenotype is alternatively activated by Th2 cell cytokines including interleukin 4 (IL-4) and plays vital roles in promoting cell growth, collagen formation, tissue repairing, and fibrogenesis (Shearer et al. 1997; Song et al. 2000; Wynn and Barron 2010; Wynn and Ramalingam 2012). Upregulation of IL-4 and induction of alveolar M2 macrophage were reported in the lung and bronchoalveolar lavage (BAL) fluid from patients with idiopathic pulmonary fibrosis (IPF), supporting that M2 macrophage is a key mediator controlling fibrogenesis and inhibition of macrophage alternative activation is one of the ideal strategies to treat pulmonary fibrosis (Prasse et al. 2006; Pechkovsky et al. 2010). The irreversible pulmonary fibrosis is destructive and often leads to progressive dyspnea and gradual deterioration in lung function (Gross and Hunninghake 2001; Meltzer and Noble 2008). Although several anti-fibrotic agents were reported to partially relieve symptoms and detain fibrosis progression, currently no pharmaceutical is clinically available to resolve established fibrotic nodules and completely cure fibrosis (Iyer et al. 1998; Demedts et al. 2005; Choe et al. 2010; Pini et al. 2010).

A breakthrough was revealed in a high-throughput proteomic study, in which apolipoprotein A-I (Apo A-I) was significantly diminished in BAL fluid from IPF patients (Kim et al. 2010). Intranasal replenishing of Apo A-I was shown to ameliorate bleomycin induced lung injury in mice. More importantly, continuing investigation using transgenic mice demonstrated for the first time that silica-induced alveolar fibrotic nodules were partially resolved by Apo A-I local overexpression (Lee et al. 2013). However, despite that Apo A-I holds exceptional anti-fibrotic potential, genetic therapy for human is ethically challenging and far from readily available. Therefore, effective and practical Apo A-I substitutes are in demand to fight against pulmonary fibrosis.

D-4F, a synthetic Apo A-I mimetic with 18 α-amino acids, was claimed safe in human clinical trials for coronary heart disease (Bloedon et al. 2008). Since Apo A-I is the major component of plasma high-density lipoprotein (HDL), D-4F was mainly...
investigated in treating atherosclerosis and cardiovascular disorders (Navab et al. 2005, 2006; Getz and Reardon 2011). To date, only a few studies have shed light on the effects of D-4F on respiratory diseases. In the asthmatic model, D-4F was shown to decrease airway hyper-responsiveness and oxidative stress (Nandedkar et al. 2011). In alveolar cells, D-4F was reported to inhibit transforming growth factor β1 (TGF-β1) induced epithelial-mesenchymal transition (EMT) (You et al. 2016). As EMT is extensively documented in the pathogenesis of pulmonary fibrosis, D-4F was speculated to exert anti-fibrotic effect similar to Apo A-I (Selman and Pardo 2003; Kasai et al. 2005; Willis et al. 2005). Given M2 macrophage and TGF-β1 are the key regulators of fibrosis and ubiquitously up-regulated in IPF patients, whether D-4F can interfere with macrophage activation and TGF-β1 expression is of particular interest.

Datta and colleagues reported that Apo A-I and mimetic 4F attenuate lipopolysaccharide (LPS)-induced inflammation by initiating functional changes in monocyte-derived macrophages (MDMs), thereby improve HDL function and inhibit atherosclerotic lesion (Navab et al. 2002; Smythies et al. 2010; White et al. 2012). Nevertheless, the milieu and timing of inflammatory events in pulmonary fibrosis are vastly different from cardiovascular diseases (Wilson and Wynn 2009). In addition, D-4F exerts superior pharmacokinetics and biosafety than its 4F counterparts due to the lack of peptidase for D-amino acids in mammalian system (Bloedon et al. 2008). To date, no study has demonstrated any observation between D-4F and fibrosis related M2 macrophages. Thus, this work was aimed to preliminarily explore the effects of D-4F on IL-4 induced macrophage alternative activation and pro-fibrotic TGF-β1 expressions, in order to shed light on the basis for future possible treatment of pulmonary fibrosis.

Materials and methods

Chemicals and reagents

Recombinant human IL-4, human TNF-α and TGF-β1 ELISA kits were purchased from R&D Systems (Minneapolis, MN). F4/80 antibody (BM8) [FITC] and CD163 antibody (6E10.1G6) [Alexa Fluor (R) 647] were purchased from Novus Biologicals (Minneapolis, MN, USA). PrimeScript cDNA synthesis kit and Premix Ex taq kit were from Takara (Dalian, China). D-4F (Ac-DWFYDVKAYFEKAFFK-NH₂) was synthesized by Scilight-Peptide (Beijing, China).

Cell lines and culture

The human acute monocytic leukemia cell line, THP-1 (The American Type Culture Collection, Manassas, VA, USA), was maintained in RPMI-1640 medium containing 10% FBS, 100 U/mL penicillin and 100 μg/mL streptomycin in a humidified 5% CO₂ atmosphere at 37°C. When 70–80% confluence was reached, cell passages were conducted. All experiments were performed using cells after three or four passages.

Alternatively activated macrophages and stimulations

Subcultures of THP-1 cells were seeded in six-well culture dishes at a cell density of 5 × 10⁵ per dish for subsequently stimulation and analysis. THP-1 cells were treated with 100 ng/mL of PMA for 48 h and stimulated with 20 ng/mL of IL-4. To investigate the effect of D-4F on M2 activation, cells were treated for 24 and 48 h with control, IL-4 (20 ng/mL) alone, or IL-4 (20 ng/mL) in the presence of D-4F (1, 5, and 10 μg/mL).

Flow cytometric analysis

Under different stimulation conditions mentioned above, cells (1 × 10⁶) were incubated with antibodies or control in dark for 30 min at 4°C. Then, the cells were washed with PBS buffer twice, fixed with 1% paraformaldehyde, and subjected to flow cytometric analysis. The data were analysed by FlowJo software (Tree Star, Ashland, OR, USA).

RT-PCR and transcriptional analysis

Primer pairs in the RT-PCR were (5’ACCAACTATTGCTTCA GCTC) and (5’CTTGACAGGACGCCAGCATCA) for TGF-β1, (5’ATCTACCTGGGAGCGTCTT) and (5’GAGTGGCCAAGAAGAAGTGG) for TNF-α, (5’GGACCTGAGCAAGAGATGG) and (5’GCACGTGTTGCGGTACAG) for β-actin. β-Actin was used as the internal control. Arithmetic formula 2⁻ΔΔCT was used to quantify the relative changes in the transcription of TNF-α and TGF-β1.

ELISA and translational analysis

Under varying conditions mentioned above, cells were harvested and centrifuged at 1000 g for 10 min, followed by ELISA analysis on collected supernatant according to the manufacturer’s instruction.
The collected data were reported as mean ± standard error of mean (SEM). Experiments were conducted in triplicates for all the quantifications. Differences among groups were analysed using one-way ANOVA test. Statistical analyses between two groups were conducted using Dunnet \( t \)-test. Significance was assumed when \( p \leq 0.05 \).

**Results**

**IL-4 induces alternative activation of PMA treated THP-1 cells**

In order to analyse the effects of D-4F on M2 macrophages, valid macrophage alternative activation was firstly established and evaluated. THP-1 cells were incubated with 100 ng/mL of PMA for 48 h to obtain MDMs. In the absence of PMA, cells were isolated and suspended with a globular morphology representing typical monocyte (Figure 1(A)). In the presence of PMA, cells were adherent and aggregated with observable pseudopodia resembling MDMs (Figure 1(B)). This transition was confirmed by flow cytometry (Figure 2(A,B)). Under the stimulation of PMA, 83.53% of the cells were identified as MDMs based on their expression of macrophage specific F4/80 antibody. The MDMs were then treated with 20 ng/mL of IL-4 for 48 h to induce alternative activation, followed by flow cytometric analysis (Figure 2(C)). In the presence of IL-4, 92.05% of the cells were characterized as MDMs, 67.67% of cells were MDMs without expression of M2 phenotypic surface marker CD163 and 24.38% of the cells were identified as M2 macrophages, leading to a 26.49% alternative activation. These results confirmed that IL-4 was able to induce alternative activation in MDMs.

**D-4F suppresses IL-4 induced alternative activation in PMA treated THP-1 cells**

Flow cytometric analysis was performed on MDMs in the presence of IL-4 (20 ng/mL) and varying concentrations of D-4F (1, 5, and 10 \( \mu \)g/mL), in order to investigate the effects of D-4F on IL-4 induced macrophage alternative activation. Under the stimulation of IL-4 and 1 \( \mu \)g/mL of D-4F, 95.93% of the cells were identified as MDMs, 82.05% of cells were non-M2 MDMs, and 13.88% of the cells showed M2 features, resulting in a 14.47% alternative activation (Figure 2(D)). In the presence of IL-4 and 5 \( \mu \)g/mL of D-4F, 93.99% of the cells were MDMs, 84.03% of cells were MDMs without CD163 expression, and 9.96% of the cells transformed into M2 macrophages, causing a 10.60% alternative activation (Figure 2(E)). When IL-4 and 10 \( \mu \)g/mL of D-4F were present, 95.39% of the cells were MDMs, 82.31% of cells were MDMs in absence of M2 characteristics,
and 10.08% of the cells were M2 macrophages, leading to a 10.57% alternative activation (Figure 2(F)). Compared to the 24.49% alternative activation when MDMs were incubated with IL-4 alone (Figure 2(C)), addition of 1, 5, and 10 μg/mL of D-4F led to a 45.38%, 59.98%, and 60.10% reduction in alternative activation, respectively. These results manifested that D-4F was able to partially suppress IL-4 induced macrophage alternative activation and indicated that the phenotypical inhibitory effects of D-4F might be saturating at a concentration of 5 μg/mL.

**D-4F increases TNF-α transcription and translation in IL-4 induced M2 macrophages**

TNF-α is a pro-inflammatory cytokine closely associated with classically activated macrophage. The expression of TNF-α may serve as an indication for the differentiation and function of macrophages. In order to evaluate effects of D-4F on the transcription and translation of TNF-α in IL-4 induced M2 macrophages, RT-PCR and ELISA analysis were performed.

THP-1 cells were treated with PMA for 48 h to induce MDMs, followed by treatment with medium control, IL-4 alone, or IL-4 and varying concentrations of D-4F (1, 5, and 10 μg/mL) for 24 and 48 h. The relative mRNA levels of TNF-α were calculated against β-actin. Apparent down-regulation of TNF-α mRNA was noted in MDMs treated with IL-4 for 24 and 48 h, indicating that the transcription of TNF-α was inhibited in IL-4 induced M2 macrophages (Figure 3(A)). When both D-4F and IL-4 were incubated with MDMs for 24 and 48 h, increases of TNF-α mRNA levels were significantly increased in IL-4 treated MDMs compared to MDMs treated with IL-4 alone. When MDMs were treated with IL-4 and 1, 5, and 10 μg/mL of D-4F for 48 h, the relative TNF-α mRNA levels increased 8%, 11%, and 16%, respectively (Figure 3(A)). These results suggested that the IL-4 induced down-regulation of TNF-α was hindered at transcription level by D-4F.

To investigate TNF-α translation, the supernatants of treated cells were collected and quantified by ELISA analysis. When MDMs were incubated with IL-4 for 24 and 48 h, TNF-α levels decreased to 256 and 388 pg/mL, respectively. The 25% and 27% reduction signified that IL-4 was able to suppress TNF-α expression and pro-inflammatory differentiation of macrophages (Figure 3(B)). When both D-4F and IL-4 were incubated with MDMs for 24 and 48 h, increases of TNF-α expression were more substantial with higher concentrations of D-4F. When MDMs were stimulated with IL-4 and 1, 5, and 10 μg/mL of D-4F for 48 h, the TNF-α concentrations were 429, 475, and 487 pg/mL, demonstrating an increase of 11%, 22%, and 26%, respectively (Figure 3(B)). These results showed that D-4F reversed IL-4 triggered TNF-α down-regulation at translation level and might possess counter effects against IL-4 during macrophage alternative activation.

**D-4F reduces TGF-β1 transcription and translation in IL-4 induced M2 macrophages**

TGF-β1 is a pro-fibrotic cytokine strongly related to alternatively activated macrophage. The expression of TGF-β1 not only reflects macrophage activation but it also initiates EMT and affects the progression of pulmonary fibrosis. Therefore, transcriptional and translational studies were conducted to determine whether D-4F has impact on TGF-β1 expression in IL-4 induced M2 macrophages.

Results from RT-PCR analysis showed that TGF-β1 mRNA levels were significantly increased in IL-4 treated MDMs compared to the control, manifesting that IL-4 was able to facilitate TGF-β1 transcription in MDMs (Figure 4(A)). When cells were treated with both D-4F and IL-4 for 24 h, the relative levels of TGF-β1 mRNA decreased with increasing concentrations of D-4F compared to cells treated with IL-4 alone. When the stimulation of IL-4 and 1, 5, and 10 μg/mL of D-4F were extended to 48 h, the relative TGF-β1 mRNA levels dropped 21, 37, and 39%, respectively (Figure 4(A)). These results indicated that D-4F obstructed the IL-4 induced TGF-β1 transcription in MDMs.
fibrotic solution. These results suggested that D-4F suppressed TGF-β1 transcription and translation in IL-4 induced M2 macrophages. Compared to the cells treated with IL-4 alone, when cells were treated with IL-4 and D-4F (1, 5 and 10 μg/mL), (A) RT-PCR showed that the relative mRNA levels of TGF-β1 decreased 9%, 15% and 16% in 24 h incubation (grey bar) and decreased 21%, 37% and 39% in 48 h incubation (black bar) and (B) ELISA analysis demonstrated that the TGF-β1 protein expression decreased 20%, 22% and 33% in 24 h incubation (grey bar) and decreased 29%, 55% and 61% in 48 h incubation (black bar). Asterisks indicate significant differences (p ≤ 0.05) when comparing to the cells treated with PMA and IL-4 (MDMs with the highest level of alternative activation).

Results from ELISA analysis illustrated that TGF-β1 translation was drastically higher in the presence of IL-4 than that in the control. When MDMs were stimulated by IL-4 for 24 and 48 h, TGF-β1 protein levels rose to 13.62 and 27.01 pg/mL, respectively, supporting the capability of IL-4 to induce the differentiation of pro-fibrotic M2 macrophages (Figure 4(B)). When both D-4F and IL-4 were incubated with MDMs for 24 and 48 h, decrease in TGF-β1 expression was more pronounced with increasing concentrations of D-4F. When MDMs were stimulated with IL-4 and 1, 5, and 10 μg/mL of D-4F for 48 h, the TGF-β1 concentrations were 19.15, 12.27, and 10.47 pg/mL, resulting in a reduction of 29%, 55%, and 61%, respectively (Figure 4(B)). These results suggested that D-4F suppressed TGF-β1 translation in IL-4 induced M2 macrophages, providing feasible anti-fibrotic solution.

Discussion

This study showed for the first time that D-4F, an Apo A-I mimic with desirable properties of pharmacokinetics and bio-safety, suppressed IL-4 induced macrophage alternative activation and TGF-β1 expression in PMA treated THP-1 cells. PMA was able to induce MDMs in THP-1 cells based on the morphological and flow cytometric characterizations. D-4F was able to prevent the expression of M2 macrophage specific surface marker CD163 on MDMs. In addition, D-4F significantly reversed the IL-4 induced down-regulation of M1 macrophage associated TNF-α and suppressed the up-regulation of pro-fibrotic TGF-β1. When IL-4 treated MDMs were incubated with high concentration of D-4F (10 μg/mL) for 48 h, the mRNA and protein levels of TNF-α increased 16% and 26%, while the mRNA and protein levels of TGF-β1 decreased 39% and 61%. When IL-4 treated MDMs were incubated with low concentration of D-4F (1 μg/mL) for 24 h, the effects were less evident. The reported results in the present study supported that D-4F might possess anti-fibrotic effects by inhibiting macrophage alternative activation and TGF-β1 expression.

D-4F was originally synthesized based on the structure of Apo A-I, aiming to reproduce Apo A-I functions and treat disorders caused by Apo A-I deficiency. Apo A-I is the major component of HDL, exerting anti-inflammatory property that is beneficial to cardiovascular diseases including atherosclerosis (Kwiterovich 1998). Accordingly, research has been intensively focused on the effects of D-4 on cardiovascular disorders. D-4F was shown to bind pro-inflammatory fatty acid hydroperoxides and oxidized phospholipids, improve anti-inflammatory functions of HDL and decrease atherosclerotic lesions (Navab et al. 2005, 2006; Anantharamaiah et al. 2007; Getz and Reardon 2011). The first human clinical trials of D-4F demonstrated that 500 mg of single D-4F administration was safe and well tolerated in patients with high-risk of coronary heart disease (Bloedon et al. 2008). This inspiring work underlies numerous possibilities for clinical applications of D-4F.

Recently, a few pioneering studies switched the focus to the effects of D-4F on respiratory diseases. D-4F was reported to decrease pulmonary inflammation, oxidative stress, and airway hyper responsiveness in experimental asthma induced by ovalbumin sensitization, hinting the application of D-4F on asthma relief (Nandedkar et al. 2011). Furthermore, the expression of TGF-β1 was disrupted by intranasal treatment of D-4F in asthmatic animal model, inferring D-4F might also act on TGF-β1 related disorders. In alveolar epithelial cells, D-4F was speculated to hold promise for the treatment of pulmonary fibrosis due to the inhibition of TGF-β1 induced EMT (You et al. 2016). In human type II pneumocytes, D-4F was shown to reduce the inflammatory responses caused by influenza infection (Van Lenten et al. 2004). Similar anti-inflammatory properties of Apo A-I mimic 4F were reported in MDMs, validating its vascular protective effects (Smythies et al. 2010). MDMs were treated with relatively low concentration of 4F for 7 d, and transcriptions of 8 phenotypical genes were measured. In 4F treated MDMs, the expression of seven genes was similar to those in M2 macrophages, while the expression of the other gene resembled that in M1 macrophage. These contradicting
results indicated that 4F treated MDMs primarily showed M2 phenotype while shared some characteristics of M1 phenotype, leading to the assumption that 4F was able to induce anti-inflammatory differentiation in MDMs (Smythies et al. 2010). However, the characteristics of D-4F cannot be simply assumed to be the same as 4F due to the different three-dimensional structures. The specific effect of D-4F on IL-4 induced macrophage alternative activation has never been addressed. In addition, the pathogenesis and microenvironment of inflammatory cardiovascular disorders is not comparable to pulmonary fibrosis.

In IPF patients, absence of inflammation was observed, and the M2 macrophages were found to be the predominant phenotype, supporting the theory that the timing of inflammatory events and M2 macrophages play determinant roles in pulmonary fibrosis (Selman et al. 2001; Wilson and Wynn 2009). Induction of M2 macrophages contributes to local TGF-β1 expression, which in turn triggers EMT and initiates fibrosis. On the contrary, the presence of phagocytic macrophages assists fibroblast clearance and decreases TGF-β1 expression (Moodley et al. 2003). Therefore, late-stage inflammation could actually be favourable in resolution of dysregulated tissue repair and fibrosis (Wilson and Wynn 2009). Theoretically, substances that can decrease the amount of M2 macrophages, induce late-stage inflammation and reduce the level of TGF-β1 could potentially be candidates for treatment of pulmonary fibrosis. The present study investigated the effects of D-4F on IL-4 induced macrophage alternative activation. D-4F was reported to directly reduce the amount of IL-4 induced M2 macrophages and TGF-β1 expression, suggesting antagonist effects against IL-4 and anti-fibrotic potential. D-4F was also shown to rectify the IL-4 induced TNF-α down-regulation, which might be beneficial to late-stage inflammation and fibrosis resolution.

Pulmonary fibrosis is a malignant and multifactorial disorder with poor prognosis, and no clinical treatment can reverse the established fibrosis once formed. Global proteomic analysis on 14 IPF patients reported significant reduction of Apo A-I in BAL fluid (Kim et al. 2010). Analysis on transgenic mice bearing human Apo A-I showed that the volume of fibrotic nodules, TGF-β1 expression and collagen deposition were distinctly reduced when Apo A-I was locally expressed 15 days after silica exposure, suggesting resolution of pulmonary fibrotic nodule (Lee et al. 2013). Although Apo A-I is hitherto the only reported substance that can partially resolve experimental lung fibrotic nodules, obtaining sufficient quantities of Apo A-I is impractical, let alone the poor pharmacokinetic properties. Instead, Apo A-I mimetic D-4F is free from intrinsic enzymatic degradation and clinically safe in human clinical trials. However, in order to be considered as a realistic therapeutic approach for pulmonary fibrosis, further studies on the side effects of D-4F and in vivo validations should be performed.

In conclusion, the present study showed that D-4F effectively suppresses IL-4 induced macrophage alternative activation and pro-fibrotic TGF-β1 expression.

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Disclosure statement

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