Original Paper

Low Molecular Weight Heparin Inhibits Circulating Fibrocytes Differentiation by Modulating Neuronal Nitric Oxide Synthase and TGF- β 1/Smad Pathway

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Key Words

Low molecular weight heparin • Circulating fibrocytes • Fibrosis • Nitric oxide synthase • Transforming growth factor - β 1

Abstract

Background/Aims: Circulating fibrocytes (CFs) have been placed at the center of a number of fibrosing conditions. Recently, attention has been drawn to the non-anticoagulant activities of low molecular weight heparin (LH), especially its anti-fibrotic effects. The purpose of this study was to investigate the effects of LH on CFs differentiation and possible underlying mechanisms. **Methods/Results:** CFs were cultured from human peripheral blood mononuclear cells and identified by dual-immunofluorescence staining. Incubation with LH inhibited CFs trans-differentiation by upregulating CD34 and downregulating pro-Collagen I and α -SMA in a concentration- and time-dependent manner, all of which were detected by flow cytometry. Similar effects were observed after incubation with L-NAME, an inhibitor of NOS. NO production was measured by Griess methods and markedly decreased in CFs treated with LH. Three NOS isoforms were assessed by western blot and nNOS was the predominant isoform involved in this process. Additionally, LH and L-NAME had similar down-regulating effects on the expression of TGF- β 1 and pSmad2/3, which indicated that TGF- β /Smad pathway might be a downstream signaling of nNOS/NO during LH treatment. **Conclusion:** These results suggested that LH could exhibit anti-fibrotic effects by inhibiting CFs transdifferentiation, in which the involvement of nNOS/NO and TGF- β /Smad pathway were identified.

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Introduction

Circulating fibrocytes (CFs), a distinct population of blood-borne cells that co-express hematopoietic cell antigens and fibroblast products, have been shown to play an important role in the generation of fibrosis in different fibrosing disorders, such as renal fibrosis, pulmonary fibrosis and cirrhosis [1-3]. Studies have demonstrated that CFs traffic to the site of tissue injury, differentiate into fibroblasts and myofibroblasts, and contribute to collagen deposition and fibrosis [4-6]. However, the regulatory processes that govern the differentiation of blood-borne fibrocytes are currently not known.

Low molecular weight heparin (LH) is produced by the depolymerization of unfractionated heparin with lower mean M_r between 3 and 6 kD and chain lengths of 12 to 18 saccharide units. Despite their similar anticoagulant activities between LH and heparin, the structure differences give LH distinct non-anticoagulant properties. Recently, attention has been drawn to the anti-fibrotic effect of LH in both animal models and human diseases [7-10]. Additionally, studies *in vitro* have reported that LH contributes to the differentiation of several types of progenitor cells [11, 12].

Based on these studies, it was hypothesized that LH might act as anti-fibrotic agent via inhibiting CFs trans-differentiation into fibroblasts and myofibroblasts. We therefore investigated the effects of different concentrations of LH on CFs differentiation and the time course of the potential effects, and the underlying signal transduction pathways.

Materials and Methods

Cell Culture and Stimulation

CFs from human peripheral blood were isolated and characterized as previously described [13]. To harvest and culture human CFs, 20 ml of venous blood was collected in a heparinized centrifuge tube (BD Biosciences) from healthy volunteers who had provided written informed consent. Peripheral blood mononuclear cells (PBMCs) were separated from whole blood by centrifugation on a Ficoll density gradient (Invitrogen, Carlsba, CA) following the manufacturer's protocol. After centrifugation at 2000g for 20 min, the cells at the interface were harvested, washed twice, and resuspended in DMEM (GIBICO, USA) containing 20% FCS (Sijiqing Biotech, China). After 5 days of culture, non-adherent cells were removed by gentle aspiration and media were replaced. Adherent cells were cultured continually for another 5-9 days for further experiments. One day before drug administration (10th day), cells were under serum starvation for 24 h in DMEM, 2% FCS, released from growth arrest by adding DMEM, 20% FCS, with various LH concentrations for various incubation times, then washed and used in next assays. CFs were divided into 5 groups. (1) Control group: DMEM containing 20% FCS without LH; (2)-(5) LH treatment group: containing 0.1, 1, 10 and 100 IU/ml LH respectively. L-NAME (1mM), a nitric oxide synthase (NOS) inhibitor, was used as the positive control. For time course assay, CFs were treated with the most effective agents for 12 h, 24 h, 48 h and 72 h, respectively.

The LH (FraxiParine, 4100 anti Xa IU/0.4mL) was purchased from Hangzhou Sanofi Minsheng limited companies.

Dual immunofluorescence and confocal microscopy

CFs were identified by means of dual immunofluorescence for CD34 and pro-collagen I (pro-Coll) expression. The cells seeded on fibronectin-coated chamber slides on 10th day were fixed with 4% paraformaldehyde for 5 min at 4°C and permeabilized with 0.1% Triton X-100 for 20 min in PBS. The cells were incubated with a polyclonal goat anti-human pro-Coll antibody (Santa Cruz, dilution 1:50) overnight at 4°C. The slides next were incubated for 1 h at room temperature with an Alexa Fluor 488-labeled rabbit anti-goat antibody (Molecular Probes, 1:100). Then the slides were incubated with a monoclonal mouse anti-human CD34 antibody (Santa Cruz, dilution 1:100) overnight at 4°C and then with an Alexa Fluor 546-labeled goat anti-mouse antibody (Molecular Probes, 1:100). This was followed by staining with DAPI (Sigma, 1:10000) for 20 min. Normal goat or mouse IgG was used instead of primary antibody as negative

control. Each step was followed by thorough washing with PBS. Confocal microscopy was performed using a fluorescence laser scanning confocal microscope (Olympus Fluoview FV 1000).

Flow cytometry

The cultured fibrocytes (1×10^6 cells) were immersed in permeabilizing solution and incubated with antibodies against CD34, pro- α 1 and α -SMA (mouse monoclonal antibody, Dako), indirectly labeled with Alexa Fluor 488 or 546 as required. All experiments included isotype controls. Cells (2×10^5) were flow cytometrically analyzed using the Cell Quest software (Becton Dickinson) and expressed as the results.

Determination of nitric oxide (NO) generation

The level of nitrite/nitrate in the conditioned medium of CFs was measured using a commercial kit (Nanjing Jiancheng Biology Graduate School, P.R. China). In brief, 50 μ l of medium or standard NaNO_2 was mixed with 50 μ l of Griess reagent in a 96-well plate. After 15 min, optical density was read in a microplate reader at 540 nm with a spectrophotometer. Each experiment was performed in triplicate. A standard curve using NaNO_2 was generated for each experiment for quantification. Results of six independent experiments were used for statistical analysis.

Western blot

The extracted cell protein of each sample (30 μ g total protein) was fractionated by sodium dodecyl sulphate (SDS) polyacrylamide gel electrophoresis and transblotted onto nitrocellulose filters. The membranes were incubated overnight at 4°C using mouse monoclonal eNOS (Santa Cruz), mouse monoclonal iNOS (Transduction Laboratories), mouse monoclonal nNOS (Transduction Laboratories), rabbit polyclonal TGF- β 1 (Beijing Zhongshan), goat polyclonal Smad2/3 (Santa Cruz), rabbit polyclonal phosphor-Smad2/3 (Santa Cruz) and mouse monoclonal β -actin (Sigma) antibodies. The membranes were then incubated for 2 h in the appropriate diluted secondary antibody. Signal was detected using an electrochemiluminescence (ECL) method. Exposed films were quantified on an automated imaging system (Eastman Kodak™, Rochester, NY, USA). A molecular weight marker (Invitrogen™, Carlsbad, CA, USA) was used to estimate the molecular weight.

Statistical analysis

Data are expressed as the means $\pm$ SEM. When analyzing three or more group, statistical significance was measured by one-way ANOVA. Student's t test was performed to compare two groups. P values less than 0.05 were considered significant.

Results

CFs morphology and identification

When cultured *in vitro*, CFs gradually displayed an adherent, spindle-shaped morphology by adoption of a plump fibroblast-like appearance. Circular or oval nucleus was observed in the center of the cells as well as filopodia of different length at both ends. CFs were identified as fibroblast-like cells double positive for CD34 and pro- α 1 (Fig. 1 A-D).

When incubated with LH, CFs exhibited distinct cellular morphologies compared with control. Increasing concentrations of LH inhibited CFs transformation into fibroblast-like cells and led to a more rounded, less mature appearance (Fig 1. F). Meanwhile, the number of CFs under LH treatment did not differ significantly from controls (all $p > 0.05$).

Effects of LH on CFs differentiation

The concentration of 1 IU/mL was selected for time course analysis. It showed that the effect of LH on CFs was time-dependent. It reached to climax after 48 h ($p < 0.05$) and presented decreasing tendency after 72 h, but there was no significant difference between 48 h and 72 h ($p > 0.05$).

We detected CFs phenotype by flow cytometry using the hemopoietic stem cell antigen CD34, the fibroblast marker pro- α 1 and the myofibroblast marker α -SMA. After

Fig. 1. Representative photomicrographs of cultured fibrocytes morphology. A-D, Fibrocytes were identified by dual-immunofluorescence staining using CD34 and pro-Collagen I. The separate markers for CD34 (red), pro-Collagen I (green) and nuclei staining (blue), and a merge picture (yellow) are shown. E-F, After 14 days in culture, more attached mononuclear cells displayed a spindle-shaped morphology (E). The LH treatment (10 IU/ml×48 h) could reduce the fibroblast-like change (F). Original magnification×400 (A-D) and×200 (E,F).

48 h incubation with a range of LH concentrations, CFs differentiation was obviously suppressed in a dose-dependent manner *in vitro*. The percentages of CD34⁺ cells were increased significantly by 34.6%, 52.7% and 48.8%, respectively, after incubation with LH at concentrations of 1, 10, 100 IU/ml (all $p < 0.05$ or 0.01) (Fig. 2). Conversely, there was a dose-dependent decrease in the number of CFs stained positively for pro-Coll, with a maximal decline of 34% in response to 10 IU/ml compared with the controls (all $p < 0.05$ or 0.01) (Fig. 2). Similar results were observed in the percentage of α -SMA⁺ cells after treated with LH, showing decreases of approximately 28%, 34% and 33% (all $p < 0.05$ or 0.01), respectively, compared with controls (Fig. 2).

As previous studies had established the notion that NO and its NOS isoforms were involved in the differentiation of stem/progenitor cells, in the current study, we have investigated the effects of L-NAME, a direct inhibitor of NOS, on CFs differentiation. Interestingly, all the three marker proteins displayed an almost similar expression pattern in L-NAME group, showing no significant difference from LH group at concentrations of 1, 10 or 100 IU/ml (all $p > 0.05$), which indicated that NOS/NO might be involved in LH-driven inhibition of CFs differentiation.

Effects of LH on NOS expression and NO production from CFs

Treatment with LH resulted in a dose-dependent reduction in the NO production from CFs (Fig. 3). A significant decline was reached at 10 IU/ml and sustained at 100 IU/ml compared with the controls (both $p < 0.01$), but no difference was observed between the two groups. The NO level either in 10 or in 100 IU/ml group was similar with L-NAME group.

Fig. 2. Effects of LH on the CFs differentiation. The percentages of CD34+, pro-Collagen I+ and a-SMA+ cells were detected by flow cytometry after CFs were treated for 48h with different concentrations of 0, 0.1, 1, 10, 100 IU/ml LH or 1mM L-NAME. * $p < 0.05$; # $p < 0.01$.

Fig. 3. Effects of LH on the NO generation from CFs. NO production was assayed by the Griess method in CFs treated for 48h with LH at the concentrations of 0, 0.1, 1, 10 and 100 IU/ml, or 1mM L-NAME. Data are expressed as the mean $\pm$ SEM (n=6). # $p < 0.01$.

Fig. 4. Effects of LH on eNOS, iNOS and nNOS protein expression in CFs. Western blot analysis were performed after CFs were treated for 48 h with 10 IU/ml LH. Normalization of expression levels was done by comparison with β -actin. Data are shown as mean $\pm$ SEM (n=5). # $p < 0.01$.

Fig. 5. Effects of LH on TGF- β , Smad2/3 and phosphor-Smad2/3 protein expression in CFs. Western blot analysis were performed after CFs were treated for 48 h with 10 IU/ml LH or 1mM L-NAME. β -actin was used as a loading control.

We next identified the NOS isoforms expressions in CFs. As shown in Fig. 4, the protein expressions of all three isoforms (eNOS, iNOS and nNOS) were identified in CFs by western blot and densitometric analysis showed significantly higher levels of eNOS and iNOS compared with nNOS (both $p < 0.05$). After treated with LH (10 IU/ml for 48 h), the level of nNOS was decreased about 50% ($p < 0.01$), whereas no significant differences were detected in eNOS and iNOS under the same incubation conditions. These results suggested that nNOS was likely the amenable isoform involved in the LH-driven suppression of CFs differentiation.

LH treatments down-regulate TGF- β 1/Smad expression

TGF- β 1 is an important fibrogenic and growth-regulating cytokine. The present study further investigated whether TGF- β 1/Smad pathway was regulated downstream of nNOS/NO in LH-mediated anti-fibrotic effect. Fig. 5 showed that LH (10 IU/ml for 48 h) significantly reduced the contents of TGF- β 1 and pSmad2/3 compared with controls (both $p < 0.05$), similar to the L-NAME group.

Discussion

To the best of our knowledge, the present study described for the first time that LH could exert its potent anti-fibrotic activities by inhibiting CFs differentiation into fibroblasts and myofibroblasts in a dose- and time-dependant manner. LH-induced suppression of CFs tras-differentiation was associated with the inhibition of nNOS/NO signaling, as evidenced by the obvious decrease in nNOS expression and subsequent NO production. Furthermore, TGF- β 1 and pSmad were markedly reduced after LH stimulation and similar results were obtained from L-NAME treatment, which indicating that TGF- β 1/Smad pathway might be involved in the downstream of nNOS/NO signaling in LH anti-fibrotic effects.

It was found that LH had many potential therapeutic benefits beyond its traditional role as an anticoagulant. Recently, both experimentally and clinically its anti-fibrotic properties have attracted particular attention. LH has been reported to have beneficial effects on inflammation and collagen deposit in several animal models [7, 8]. Moreover, clinical data suggested that LH was protective against liver fibrosis in patients with chronic hepatitis [9, 10]. However, although LH has been shown to have protective effects against fibrogenesis, the underlying mechanisms for the anti-fibrotic effects are far from being understood.

Fibrocytes are circulating bone marrow-derived progenitors that co-express haematopoietic stem-cell antigens, markers of the monocyte lineage and fibroblast products. There has been significant evidence from across different subspecialties of medicine implicating these cells in different tissue-specific or organ-specific fibrosing disorders [1-3]. CFs comprise 0.1-0.5% of non-erthrocytic cells in peripheral blood and display an adherent, spindle-shaped morphology when cultured *in vitro*, which consistent with our study [14]. CD34⁺ fibrocytes undergo morphological and phenotypic alterations characterized by the adoption of a plump fibroblast-like appearance and loss of CD34⁺ expression accompanied by a gain of α -SMA expression [15]. In our study, we found LH exerted a pronounced inhibitory effect on CFs differentiation into fibroblasts and myofibroblasts, reserving its haematopoietic stem cells phenotype (CD34⁺). Fibroblasts have been shown to exhibit increased collagen expression and other matrix components in certain fibrotic disease states. Myofibroblasts are the cell type that generates and deposits collagen rich pathological extracellular matrix leading to irreversible fibrosis and causing organ dysfunction. There is increasing evidence that CFs contribute to the new population of myofibroblasts that emerge at the tissue site during the process of fibrosis [5, 6, 15]. Moreover, studies *in vitro* have reported that LH contributed to modulating the differentiation of progenitor cells, such as endometrial stromal cells and osteoblast [11, 12]. Taken together, these findings suggest that LH may function as anti-fibrotic agent by strong inhibition of CFs differentiation, which supporting the potential therapeutic benefits of LH in fibrosing diseases.

NO has been widely studied due to its significant importance to human cell physiology, including cell proliferation and differentiation. NO is synthesized from L-arginine by three isoforms of NOS: neuronal NOS (nNOS), inducible NOS (iNOS), and endothelial NOS (eNOS). In our previous study, it was demonstrated that eNOS/NO was involved in the Ang(1-7) induced suppression of CFs proliferation and apoptosis [13]. But there is a dearth of studies regarding all NOS isoforms expression pattern in CFs. In the present study, we identified all three NOS isoforms in CFs by western blot and relatively higher expression of eNOS and iNOS. Recently, NO has been shown to play a protagonist role in the therapeutic benefits of heparin and LH. Lakshimi et al reported that LH would be more effective in the treatment of burn patients through NOS inhibition [16]. Another report also showed that heparin significantly ameliorated lung injury via the inhibition of NOS expression [17]. In this study, we found that LH significantly reduced the expressions of nNOS and subsequent NO production in cultured CFs. Moreover, as for the effects of L-NAME (a NOS inhibitor) treatment, blockade of NO biosynthesis could suppress the CFs differentiation in a compatible pattern such as observed in LH treated groups. These results confirmed previous reports demonstrating that reduction in NO production resulted in an increased number of early haematopoietic

progenitors in nNOS knockout mice [18, 19]. It had also been demonstrated that exposure to chemical donors of NO could influence the extent of CD34⁺ bone marrow cells maturation *in vitro* [20]. Thus nNOS/NO may be acting as a negative regulator of haematopoietic progenitor cells differentiation [21]. Taking all these results together, it is concluded that nNOS may be the predominant isoform involved in LH-induced inhibition of CFs differentiation toward the fibroblast or myofibroblast phenotype. Nevertheless, the precise mechanism by which LH inhibits the nNOS/NO pathway needs to be further elucidated.

TGF- β 1, a central mediator of fibrogenesis, has been shown to be the most important cytokine for the trans-differentiation of fibroblasts to myofibroblasts that exhibit increased α SMA staining, elevated collagen secretion, and increased stress fibers [22]. TGF- β 1 stimulates collagen synthesis via the phosphorylation of Smad 2 and Smad 3 [23]. Down-regulation of TGF- β 1 expression and modulation of TGF- β /Smad signaling may be effective in preventing organ fibrosis [24]. Investigators have previously demonstrated that TGF- β 1 overexpression has been associated with enhanced myofibroblast differentiation [25, 26]. In our data it was observed that the expressions of TGF- β 1 and p-Smad2/3 were markedly decreased in cultured CFs under LH treatment. These findings were consistent with a recent report which demonstrated that LH showed anti-fibrotic effect via inhibition of the TGF- β /Smad pathway [15]. Moreover, the present study revealed a similar expression profile for TGF- β 1 and p-Smad2/3 between LH and L-NAME treatment group. In such contexts, it is possible to suggest that TGF- β /Smad pathway may be a downstream signaling of nNOS in LH-stimulated CFs.

In conclusion, the present study shows a part of the mechanism of how LH exhibits anti-fibrotic effects in fibrogenesis. These data suggest for the first time that LH inhibits CFs differentiation through suppression of nNOS and its production NO, accompanied with down-regulation of TGF- β /Smad pathway. Further studies are necessary to identify the molecules involved in modulation between LH and fibrocytes that are required for fibrocytes maturation.

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