Original Article

In vitro Effects of Nerve Growth Factor on Cardiac Fibroblasts Proliferation, Cell Cycle, Migration, and Myofibroblast Transformation

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Abstract

Background: Recent research indicates that nerve growth factor (NGF) promotes cardiac repair following myocardial infarction by promoting angiogenesis and cardiomyocyte survival. The purpose of this study was to investigate the effects of NGF on cardiac fibroblasts (CFs) proliferation, cell cycle, migration, and myofibroblast transformation *in vitro*.

Methods: CFs were obtained from ventricles of neonatal Sprague-Dawley rats and incubated with various concentrations of NGF (0, 0.01, 0.1, 1, 10, and 100 ng/ml; 0 ng/ml was designated as the control group). Cell proliferation and cell cycle of the CFs were measured by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay and flow cytometry (FCM), respectively. A cell scratch wound model and transwell were carried out to observe effects of NGF on migration of CFs after 24 h of culture. Real-time polymerase chain reaction (RT-PCR) and Western blotting were used to measure α-smooth muscle actin (α-SMA) at mRNA and protein levels after CFs were incubated with various concentrations of NGF.

Results: Expression of α-SMA measured by RT-PCR and Western blotting significantly increased in the 1 and 10 ng/ml NGF groups ($P < 0.05$). Absorbance values of CFs showed that NGF did not influence the proliferation of CFs (The A_{490} values were 0.178 ± 0.038, 0.182 ± 0.011, 0.189 ± 0.005, 0.178 ± 0.010, 0.185 ± 0.025, and 0.177 ± 0.033, respectively, in the 0, 0.01, 0.1, 1, 10, and 100 ng/ml NGF groups [$P = 0.800$, 0.428, 0.981, 0.596, and 0.913, respectively, compared with control group]), and FCM analysis showed that the percentage of CFs in G0/G1, S, and G2/M phases was not changed ($P > 0.05$). The cell scratch wound model and transwell showed that CFs migration was not significantly different ($P > 0.05$).

Conclusion: NGF induces myofibroblast transformation but does not influence proliferation, cell cycle, or migration of CFs *in vitro*.

Key words: Cell Cycle; Cell Movement; Cell Proliferation; Fibroblasts; Myofibroblasts; Nerve Growth Factor

Introduction

Cardiac fibroblasts (CFs) are the most abundant cell type in the heart and play a key role in maintaining cardiac structure and function by controlling proliferation and extracellular matrix (ECM) turnover. CFs are sources and targets of cardiac fibrosis during pathological myocardial remodeling such as myocardial infarction (MI) and hypertension.[1-3] Fibroblast proliferation, migration, and conversion to a myofibroblast phenotype in response to a variety of cytokines are related to cardiac injury and disease (such as transforming growth factor-β, connective tissue growth factor, platelet-derived growth factor, angiotensin II, endothelin-1, tumor necrosis factor-α, interleukin-6, and oxidative stress and mechanical stretch).[4,5]

Nerve growth factor (NGF) is a polypeptide that plays an important role in growth, differentiation, and survival of neurons. The biological activity of NGF is mediated by the tyrosine kinase receptor (trkA) and the low-affinity glycoprotein receptor p75 presenting on the surface of responsive cells.[6-8] NGF is expressed in normal hearts and upregulated following MI coupled

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with the regeneration of cardiac sympathetic nerves and heterogeneous innervation, which may result in ventricular tachycardia, ventricular fibrillation, and sudden cardiac death.[9,10] It was recently found that NGF promotes cardiac repair following MI by promoting angiogenesis and cardiomyocyte survival,[11] but little is known about the effects of NGF on the functions of CFs. Therefore, we aimed to investigate the *in vitro* effects of NGF on the profibrogenic properties of CFs in this study.

Methods

Cell culture

CFs were obtained from the ventricles of neonatal Sprague-Dawley rats (1–3 days old) by the trypsin digestion method and characterized as previously described.[12] All experiments were performed in cells of the third to sixth passage after starvation in serum-free Dulbecco's Modified Eagle Medium (DMEM) for 24 h. Experimental procedures and protocols were approved by the Animal Care and Use Committee of Guangzhou University of Chinese Medicine and complied with the guidance suggestion of caring laboratory animals of the Ministry of Science and Technology of China.

Cell proliferation assay

Cell proliferation was evaluated by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Briefly, CFs were plated on 96-well plates (5000 cells/well), then starved by serum deprivation for 24 h, and treated with various concentrations (0, 0.01, 0.1, 1, 10, and 100 ng/ml; 0 ng/ml was designated as the control group) of NGF (R and D Systems, USA) in DMEM containing 0.5% FBS for 24 h. MTT reagent (20 μl) (Sigma, USA) was added to wells and incubated for 4 h. The cells were dissolved in DMSO, and optical density (*A*) was measured at 490 nm.

Cell cycle analysis

CFs were plated on 6-well plates at a density of 8×10^4 cells/well, 70–80% confluence and treated with various concentrations of NGF. After 24 h, cells were collected and washed one time with phosphate-buffered saline (PBS). Reagent A (1 ml) and reagent B (10 μl) (MultiSciences Biotech Co., China) were added and blended by vortexing for 5–10 s and incubated for 30 min at room temperature. Cell cycle distribution was then analyzed by flow cytometry (FCM) (Becton, Dickinson and Company, USA).

Cell migration assay

Cell migration was detected by two methods: scratch wound healing assay and transwell migration assay.[13] CFs (8×10^4) were cultured in 6-well plates until 70–80% confluence. The media were then replaced with serum-free DMEM to inhibit cell proliferation. The scratch wound was introduced using a blue pipette tip. The cultures were washed twice with PBS to remove debris and reincubated with serum-free media with vehicle or various NGF concentrations for 24 h. The images of scratch wounds of each culture for three independent repeats were captured by an Olympus 1X71 camera (Olympus, Japan) at 0 and 24 h time points during cell migration. Cells migrating into the wound site were quantified and compared with the control group. The original magnification of each image was ×40.

For transwell migration assay, serum-starved CFs (10^5) were loaded into the upper chamber of the migration apparatus (Corning, USA). DMEM containing 0.5% FBS was introduced into the lower chamber as a chemotactic stimulus. NGF at various concentrations (0, 0.1, 1.0, 10, and 100 ng/ml) was added to the lower chambers of the experimental wells. After incubation for 24 h at 37°C in a tissue culture incubator, nonmigrated cells remaining on the upper surface of the membrane were removed with a cotton swab and migrated cells were stained with 0.1% crystal violet for 30 min. Migrated cells were visualized and counted with a microscope at magnification ×200. Five fields were counted on each membrane. This analysis was performed in triplicate for each experimental condition. Migration was expressed as the mean value of the total number of migrated cells per field.

RNA isolation and real-time polymerase chain reaction analysis

After being treated with either 0, 0.01, 0.1, 1.0, 10, or 100 ng/ml NGF for 24 h, total RNA was extracted from the CFs using Trizol reagent according to the manufacturer's instructions and quantified using ultraviolet spectrophotometry. cDNA was generated from 1 mg total RNA using an AMV Reverse Transcriptase Kit. Real-time polymerase chain reaction (PCR) was performed in an Applied Biosystems 7300 Fast real-time PCR System (Foster City, CA, USA) with SYBR Green PCR Master Mix. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA amplified from the same samples served as an internal control. The relative expression of each targeted gene was normalized by subtracting the corresponding GAPDH threshold cycle (Ct) values using the $2^{-\Delta\Delta CT}$ comparative method. The sequences of all primers used in this work were as follows: α-SMA: forward primer: AGCCAGTCGCCATCAGGAAC; reverse primer: GGGAGCATCATCACCAGCAA. GAPDH: forward primer: GCCCAGCAAGGATACTGAGA; reverse primer: TGGTATTCGAGAGAAGGGAGG.

Protein extraction and Western blotting analysis

Total CFs protein was isolated with RIPA lysis buffer (Sigma Aldrich, St. Louis, MO, USA). Protein concentrations were measured through BCA assay (Thermo Scientific, Waltham, MA, USA). Proteins were fractionated on a 10% polyacrylamide gel (Bio-Rad, Hercules, CA, USA), blotted onto nitrocellulose membrane, and probed with antibodies specific for α-SMA (Santa Cruz Biotechnology, Dallas, TX, USA). Signals were detected using ECL (Thermo Scientific, Waltham, MA, USA).

Statistical analysis

Data were expressed as the mean ± standard error (SE) of at least three independent experiments unless otherwise

stated. The software of SPSS 13.0 for Windows (SPSS Inc., IL, USA) was used for analysis. Differences among groups were tested by one-way analysis of variance (ANOVA). $P < 0.05$ was considered statistically significant.

Results

Effect of nerve growth factor on cardiac fibroblasts α-smooth muscle actin expression

The expression of a-SMA mRNA and a-SMA protein significantly increased upon 24 h treatment with 1 and 10 ng/ml NGF [Figures 1, 2a and 2b].

Effect of nerve growth factor on cardiac fibroblasts proliferation

The various doses of NGF for 24 h had no effect on the proliferation of fibroblasts isolated from neonatal rat heart. The A_{490} values were 0.178 ± 0.038, 0.182 ± 0.011, 0.189 ± 0.005, 0.178 ± 0.010, 0.185 ± 0.025, and 0.177 ± 0.033, respectively, in the 0, 0.01, 0.1, 1, 10, and100 ng/ml NGF groups ($P = 0.800$, 0.428, 0.981, 0.596, and 0.913, respectively, compared with control group).

Effect of nerve growth factor on cardiac fibroblasts cell cycle

The cell cycle of CFs was determined by FCM. There was no significant difference in the percentage of cells in the G(0)/G(1) phase when compared to the control group (82.332% ± 4.001%, 82.298% ± 2.316%, 81.670% ± 2.469%, 80.628% ± 1.009%, and 80.652% ± 2.382% in the 0.01, 0.1, 1, 10, and 100 ng/ml NGF groups and 82.203% ± 3.002% in control) (P = 0.940, 0.956, 0.756, 0.362, and 0.370, respectively, compared with control group).

There was also no significant difference in the percentage of cells in the S phase when compared to the control group (10.283% ± 3.415%, 10.005% ± 3.145%, 10.328% ± 2.759%, 10.577% ± 2.665%, and 11.176% ± 3.600% in the 0.01, 0.1, 1, 10, and 100 ng/ml NGF groups and 10.710% ± 3.781% in control) (P = 0.838, 0.735, 0.854, 0.949, and 0.823, respectively, compared with control group).

Finally, there was no significant difference in the percentage of cells in the G(2)/M phase when compared to the control group (7.385% ± 1.446%, 7.697% ± 2.044%, 8.002% ± 1.261%, 8.795% ± 1.806%, and 8.172% ± 1.278% in the 0.01, 0.1, 1, 10, and 100 ng/ml NGF groups and 7.087% ± 1.941% in control) (P = 0.779, 0.567, 0.392, 0.117, and 0.312, respectively, compared with control group).

Effect of nerve growth factor on cardiac fibroblasts cell migration

In some conditions, cardiac fibrosis was associated with increased migration of CFs. A scratch assay was used to evaluate the potential modulation of the migratory ability of CFs by NGF. A denuded area was introduced in the center of confluent cultured fibroblasts, and the ability of fibroblasts to migrate into the denuded area was measured following the treatment of the cells with different doses of NGF. Over a 24 h period, rat CFs demonstrated no significant distance in migratory ability following treatment with the different doses of NGF used in the present study [Figure 3] (P = 0.523, 0.658, 0.568, 0.299, and 0.650, respectively, compared with control group).

The Boyden chamber assay was used to examine the effect of NGF on CFs migration. After 24 h incubation, CFs were observed on the bottom side of the polyethylene terephthalate membranes in NGF-treated groups (0.01, 0.1, 1, 10, and 100 ng/ml), and there was no significant difference compared with the control group (P = 0.737, 0.436, 0.269, 0.504, and 0.780, respectively, compared with control group). This indicated that NGF did not affect the ability of CFs to invade across the layer [Figures 4 and 5].

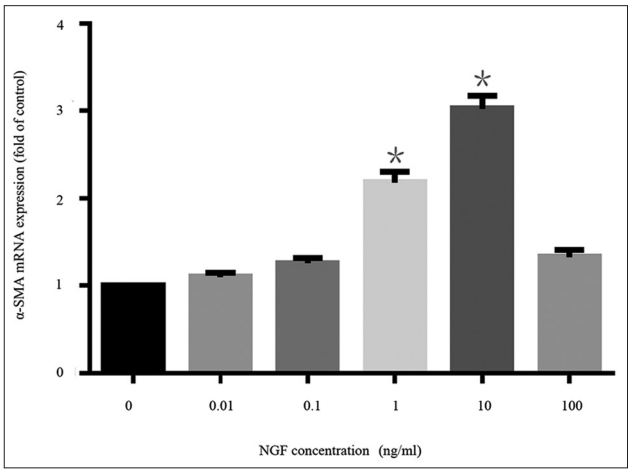

Figure 1: Real-time polymerase chain reaction of α-SMA mRNA expression. 0.01, 0.1, 1, 10, and 100 ng/ml NGF effect on cardiac fibroblasts ($n = 3$). P = 0.586, 0.057, 0.000, 0.000, and 0.143, respectively, compared with control group (0 ng/ml NGF). *$P < 0.05$ compared with control group. α-SMA: α-smooth muscle actin; NGF: Nerve growth factor.

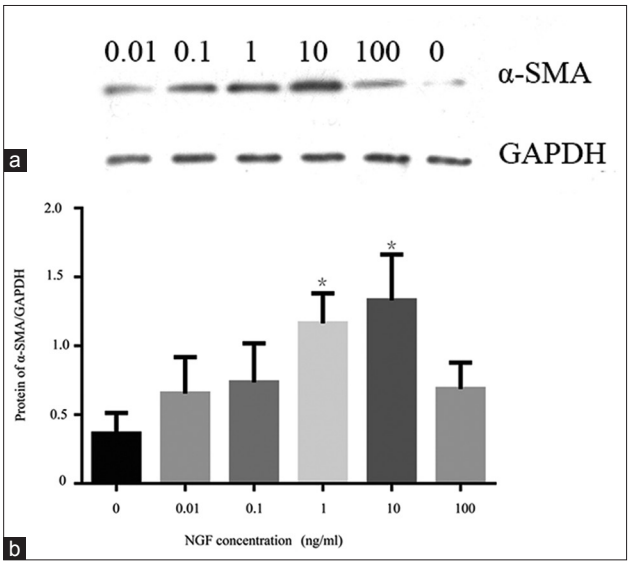

Figure 2: (a and b) Western blotting of α-SMA expression. 0.01, 0.1, 1, 10, and 100 ng/ml NGF effect on cardiac fibroblasts ($n = 3$). P = 0.268, 0.383, 0.011, 0.028, and 0.332, respectively, compared with control group (0 ng/ml NGF). *$P < 0.05$ compared with control group. α-SMA: α-smooth muscle actin; NGF: Nerve growth factor.

Discussion

Cardiac fibrosis is a common pathological process shared by multiple major heart diseases. Because CFs/myofibroblasts are the primary cells responsible for ECM deposition and turnover, it is important to understand the regulation of the function and gene expression of these cells. Myofibroblasts are not usually found in healthy cardiac tissue; however, on injury, myofibroblasts appear in the myocardium and participate in the cardiac wound healing response. Several recent reports indicated that NGF exerted a variety of effects on peripheral tissues, including fibrosis,[14-16] suggesting that NGF may be a target for cardiac fibrosis.

In our study, we found that NGF induces the expression of a-SMA in the 1 and 10 ng/ml groups, indicating their phenotype changes into myofibroblasts. These data show a direct pro-fibrogenic effect of NGF on CFs. Previous studies also indicated that NGF induced lung and skin fibroblast a-SMA expression through trkA.[14] Interestingly, the expression of a-SMA was lower in the 100 ng/ml concentration compared to that of the 10 ng/ml concentration. We speculate that (1) NGF increases a-SMA expression through the specific and unique binding to $trkA^{NGFR}$ (specific receptor of NGF) but not $p75^{NTR}$ (the pan-neurotrophin receptor); and (2) high concentrations of NGF binding with the low-affinity glycoprotein receptor $p75^{NTR}$ decrease a-SMA expression.[17] The expression of $p75^{NTR}$ by myofibroblasts seems to play a switch-off effect in the further differentiating action of NGF. NGF plays a modulatory, rather than an exclusive, stimulatory effect on the fibrotic process[18,19] so that the 100 ng/ml group of NGF decreased a-SMA expression.

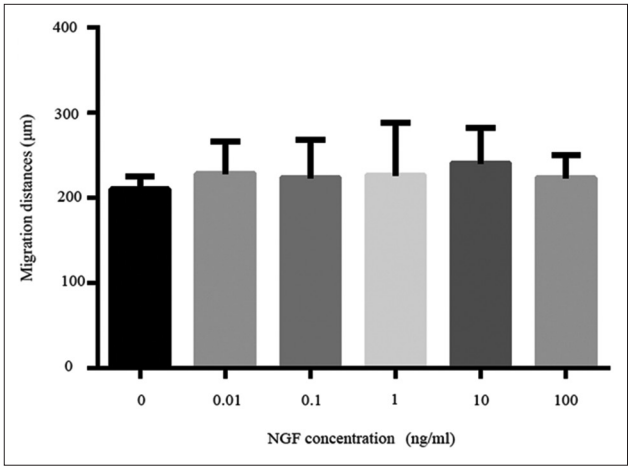

Figure 3: Scratch assay of migration distances of cardiac fibroblasts in different doses of NGF group (0.01, 0.1, 1, 10, and 100 ng/ml) compared with control group (0 ng/ml NGF), all $P > 0.05$. NGF: Nerve growth factor.

We found that NGF did not influence proliferation, cell cycle, or migration of CFs *in vitro*. Micera *et al*.[14] reported that NGF did not influence either skin or lung fibroblast proliferation, collagen production, and metalloproteinase production or activation. In contrast, NGF induced lung and skin fibroblast migration and a-SMA expression and collagen contraction. Gan *et al*.[16] found that NGF did not influence proliferation, mitotic cycle, and collagen synthesis of human dermal fibroblasts but significantly enhanced migration *in vitro* model of wounded fibroblasts. We believe that the differences may be caused by different species and tissue cell origin.

NGF concentration is higher in heart tissue than that in serum in physiological conditions or a pathological state. We chose the dose of NGF (0, 0.01, 0.1, 1, 10, and 100 ng/ml) to carry out our study according to previous studies.[14,16] The NGF content in the sera from normal subjects is about 200 pg/ml[20] and 50 pg/ml in rats; after MI, it increased to 350 pg/ml.[21] In a dog model of MI, it was found that both NGF protein and mRNA levels increased at the infarcted site and at the noninfarcted left

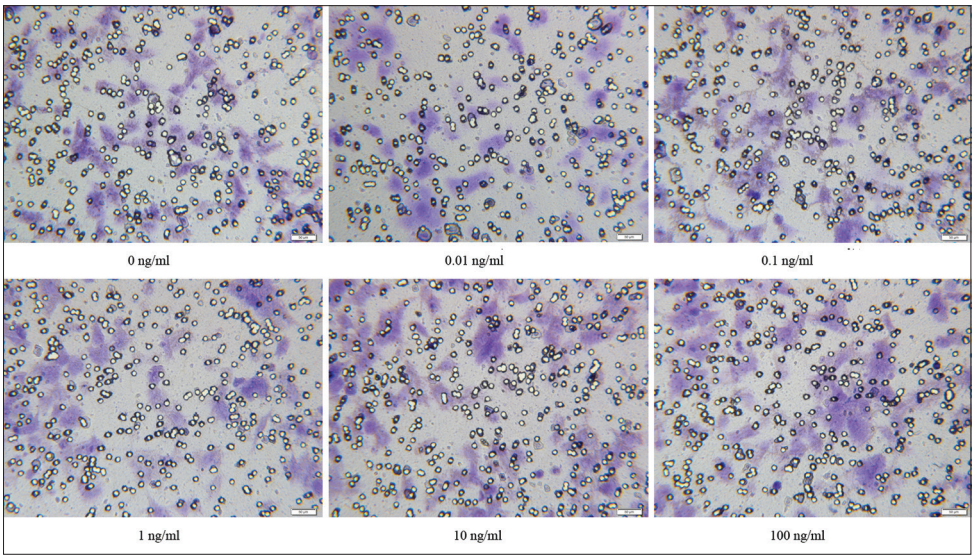

Figure 4: Cell numbers of cardiac fibroblasts migration in different groups by transwell (0, 0.01, 0.1, 1, 10, and 100 ng/ml nerve growth factor) (original magnification ×200).

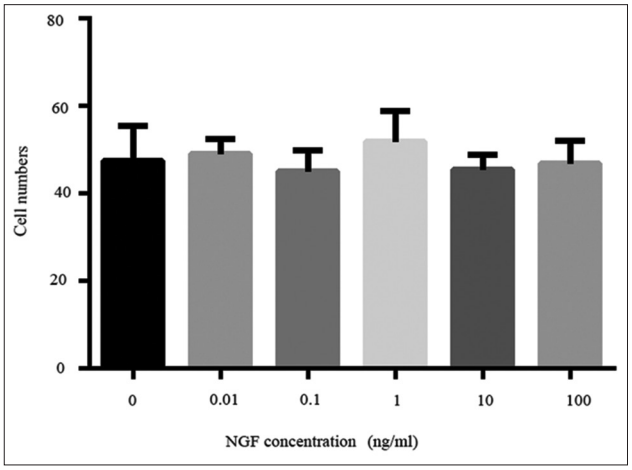

Figure 5: Cell numbers of cardiac fibroblasts migration in different groups by transwell (0.01, 0.1, 1, 10, and 100 ng/ml NGF) compared with control group (0 ng/ml NGF), all $P > 0.05$. NGF: Nerve growth factor.

ventricle free wall. NGF concentration in the coronary sinus is higher than in the aorta immediately after MI. This suggests that the heart can synthesize NGF and release it into serum.[9]

In conclusion, these data show a direct pro-fibrogenic effect of NGF on CFs and therefore NGF may be a target for cardiac fibrosis. The limitation of our study is that the detailed mechanisms underlying these effects and the chronic exposure of NGF on CFs were not explored.

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Conflicts of interest

There are no conflicts of interest.

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神经生长因子对离体心脏成纤维细胞增殖、细胞周期、迁移以及肌成纤维细胞转化的影响

摘要

背景：最近的研究表明，神经生长因子 (NGF) 通过促进血管生成、心肌细胞存活而促进心肌梗死后心脏修复。本研究旨在探讨 NGF 对心肌成纤维细胞 (CFs) 增殖、细胞周期、迁移及肌成纤维细胞转化的影响。

方法：取新生大鼠心室培养CFs。以不同浓度的 NGF (0、0.01、0.1、1、10、100 ng/ml) 孵育CFs，MTT 法和流式细胞仪 (FCM) 分别测定细胞增殖和细胞周期。采用细胞划伤模型和 Transwell法，观察不同浓度NGF培养24小时后对CFs迁移的影响。采用RT-PCR和免疫印迹法分别测定CFs中肌动蛋白 (α-SMA) 在不同浓度NGF孵育后的mRNA和蛋白水平。

结果： RT-PCR和Western blotting检测提示1、10ng/ml NGF组CFs中α-SMA的表达显著增加（$P<0.05$）。MTT法测量各组CFs的吸光度值，结果显示NGF对CFs增殖无作用（$P>0.05$），流式细胞仪检测各组CFs细胞G0/G1期、S期、G2/M期，结果无明显变化（$P>0.05$）。细胞划伤模型和Transwell表明不同组CFs细胞迁移无统计学差异（$P>0.05$）。

结论：NGF可促进肌成纤维细胞转化，但对心脏成纤维细胞增殖、细胞周期、迁移无影响。